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The biosynthesis of pseudomonic acid.

THE BIOSYNTHESIS OF PSEUDOMONIC ACID

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A thesis submitted as required for the degree of Doctor of Philosophy
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ABSTRACT

This thesis is divided into three chapters. These are all self-contained, including relevant references and experimental details, as well as results and discussion. The numbering of compounds, however, is continuous throughout the text.

Chapter one introduces the role of metabolism in gathering energy for biological processes from the environment. Phototrophic mechanisms are briefly mentioned, with primary chemotrophic metabolism being described in more detail. The methodology of biosynthetic investigations is introduced, with respect to the elucidation of the stereochemical course of fatty acid biogenesis. Nmr spectroscopic methods are described, prior to the discussion of early studies regarding polyketide biosynthesis, a secondary metabolic process.

The antibiotic pseudomonic acid is introduced in chapter 2. Work leading to the discovery of this natural product, and subsequent investigations into the biological action and clinical applicability of the metabolite is detailed. Early studies regarding the biogenesis of this entity are discussed. Investigations into the biosynthetic origins of a C_3 moiety within the molecule are presented. An hypothesis invoking the intermediacy of two distinct entities in the biogenesis of pseudomonic acid is proposed, and studies directed towards verification of this are described in both whole cell and cell free systems.

The polyketide biosynthetic pathway is further discussed in chapter three, with reference to monic acid (a constituent of pseudomonic acid) Experiments directed towards verifying a processive biosynthetic origin for this moiety are detailed, including the development of syntheses towards labelled compounds with respect to such experiments. Studies directed towards the elucidation of the differing origins of the branching methyl units within monic acid are detailed.

DECLARATION

I declare that, unless where acknowledged within the text, the work described throughout this thesis is that carried out solely by myself at the University of Bristol and SmithKline Beecham Pharmaceuticals (Brockham Park) between January 1990 and September 1992.

Martin J. Sugden.

ACKNOWLEDGEMENTS

I am indebted to Professor Tom Simpson for the creation of this project, and his subsequent help and encouragement. Thanks must also go to Dr. Keith Baggeley for similar support during my industrial placement. Funding from both the SERC and SmithKline Beecham was gratefully received.

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As always, the emotional support for this work is the most appreciated. For the odd 'swift half' and associated nights of light relief I must thank past and present members of the Simpson group, and the Lee-Sweeney-Willis possee.

My parents, who have supported me throughout the last six years, both in Bristol and Leicester, are whole-heartedly thanked for this, and for the entire 24 years of my existence.

Finally, to Helen. Without you this would not be. We had too much too soon. Those 16 months were the most memorable of my life. That time was not on our side, that we could not see it through together; this is the source of immense sorrow. You will always be special.

"What would the world be, once bereft
Of wet and of wildness? Let them be left,
O let them be left, wildness and wet;
Long live the weeds and the wildness yet."

'Inversnaid'
Gerald Manley Hopkins.

"Life must be lived forwards,
but can only be understood backwards"
Kierkegaard

CONTENTS

ABBREVIATIONS

The following abbreviations are used within the text:

Ac	Acetyl
ACP	Acyl carrier protein
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
br	Broad
Bu	Butyl
CoA	Coenzyme A
cP_{450}	Cytochrome P_{450} oxidase
D	Deuterium
d	Doublet
δ	Chemical shift
DCC	N,N'-dicyclohexycarbodiimide
DIBAL	Diisobutyl aluminium hydride
DMAP	N',N'-dimethylaminopyridine
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminotetraacetic acid (di-sodium salt)
EE	(1-ethoxy)ethoxy
Enz	Enzyme (bound intermediate)
Et	Ethyl
FAS	Fatty acid synthase
FAD	Flavin adenine dinucleotide (oxidised form)
$FADH_2$	Flavin adenine dinucleotide (reduced form)
gc	Gas chromatography
h	Planck's constant
HMDS	1,1,1,3,3,3-Hexamethyldisilazide
HPLC	High performance liquid chromatography
Hz	Hertz
J	Coupling constant
LDA	Lithium diisopropylamide
m	Multiplet
max	Maxima
Me	Methyl
NAC	N-acetylcysteamine

NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nmr	Nuclear magnetic resonance
υ	frequency
Ph	Phenyl
PKS	Polyketide synthase
PLD	Pig liver dehydrogenase
Pn	Pentyl
q	Quartet
s	Singlet
SAM	S-adenosylmethionine
t	Triplet
THF	Tetrahydrofuran
THP	Tetrahydropyran
TLC	Thin layer chromatography
TPP	Thiamine pyrophosphate
Tris	Tris[hydroxymethyl]aminomethane
t-RNA	Transport ribonucleic acid
UV	Ultra-violet
Vit	Vitamin

CHAPTER ONE

METABOLIC PROCESSES

1.1: LIFE.

1.1.1: Introduction

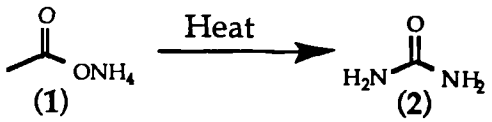
Since time immemorial the origin, function and modus operandii of life has been the source of both enchantment and perplexion for the human mind. Throughout the known world explanations for the existence of consciousness have been forwarded in terms of omniscient supra-universal Deities upon whose placation rests our continued existence.

In the 'Western world' the Judaeo-Christian philosophy of the Genesis has been the dominant creed of thought and inspiration. Indeed, the deep belief in the absoluteness of such ideas led one Irish Bishop to decree the Universe to have been created at 6am on Sunday, October 23rd, 4004 B.C.[1]

Mankind is an inately curious creature, however, and the need to inspect such philosophical pontification has led to the refinement and creation of new theories concerning the existence of life. As a result of this, the Greek teachings of Pythagoras were resurrected in the 15th century by Copernicus,[2] whose heliocentric heresies can be said to mark the conception of modern scientific thought.

Since then the development of the scientific method by Galileo and contemporaries has led to discoveries that have sought to demystify much of the physical world, and replace the pious monopoly of the clergy in explaining the enigmas of existence. Thus Newton's apple, Foucault's pendulum and Schrödinger's cat have passed into folk-lore as Physics vied with Theology for the paramount position. However, despite these and other successes, theological thought is still the dominant force in many parts of the world.

Practitioners of the chemical sciences were somewhat belated in relinquishing the influence of supra-natural forces, however. It was not until 1828, when Wöhler demonstrated that the pyrolysis of ammonium acetate (**1**) led to formation of urea (**2**), as shown in scheme 1.1.1, that the vitalist doctrine of organic chemistry was laid to rest.[3] As a result of this, new avenues of scientific thought were opened up including the study of the chemistry of life itself.



(1) (2)

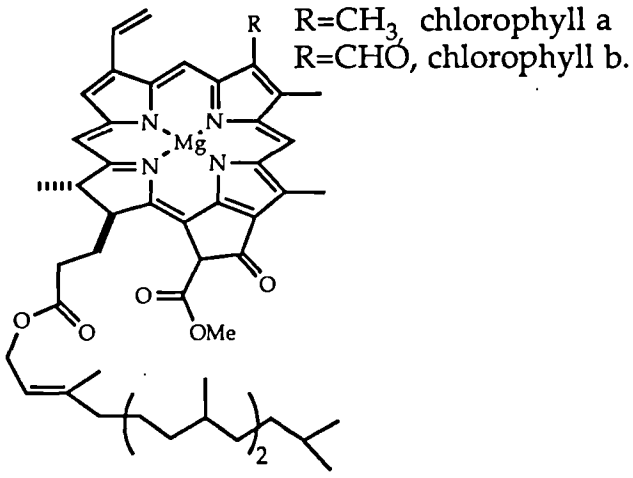
Scheme 1.1.1: Wöhler's synthesis of urea (2)

The chemical processes which underpin the propagation of life have been the subject of much scrutiny. The introduction of evolutionary theories of natural selection by Darwin[4] added impetus to such studies leading ultimately to the solution of the genetic code and the structure of DNA.

1.1.2: The energy for life.[5]

As with all physical processes the continuation of life requires a readily available source of energy. Biological processes utilise energy in chemo-electric form and two basic mechanisms for the extraction of useful energy from the environment have evolved.

As the powerhouse of the Solar System the energy of the Sun is the spark of life for phototrophic species. These trap insolation via chlorophyll (3), causing an electron potential gradient which ultimately leads to the reduction of carbon dioxide by water to form carbohydrate and oxygen. This process is called photosynthesis.[6]



Chlorophyll (3)

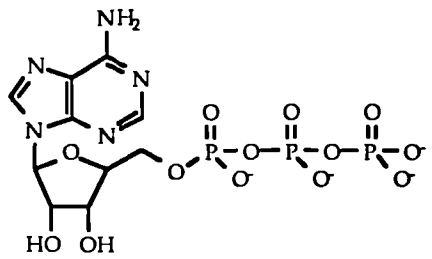
The second mechanism, chemotrophy, involves the oxidation of available foodstuffs to carbon dioxide. These foodstuffs are frequently the end product of photosynthetic processes, thus demonstrating how life itself is a further expression of the first law of thermodynamics.

The chemistry of life that leads to the generation, storeage and utilisation of appropriate energy forms is called metabolism. Two broad classes of have been identified:

a) Primary Metabolism: That which is essential for the appearance and propagation of life, and varies little across the species.

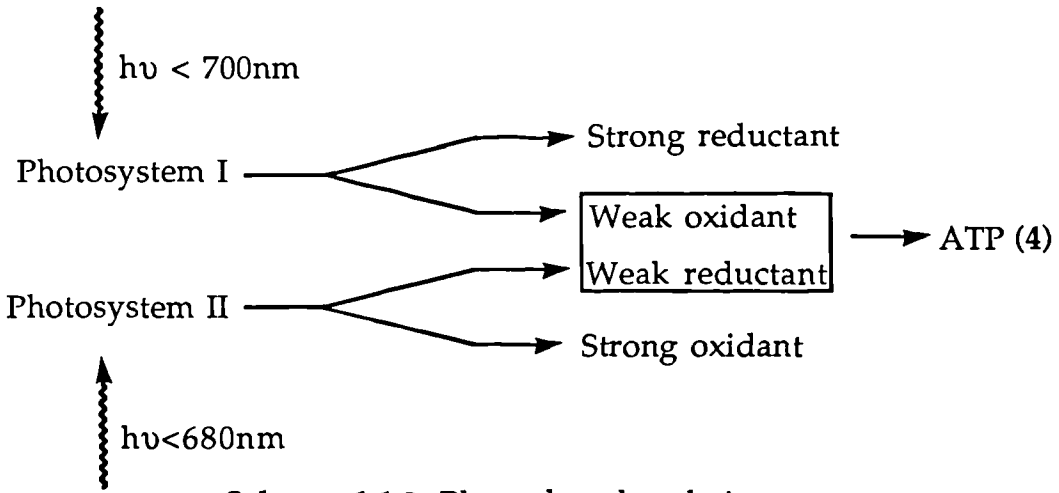
b) Secondary Metabolism: That which is not necessary for the continuance of life, and is frequently restricted to one species, or even a strain of a species.

Regardless of the mechanism of energy entrapment, all life forms store energy for immediate retrieval by means of adenosine triphosphate (4).



Adenosine tri-phosphate (4)

Phototrophic species synthesise ATP (4) by the use of two finely balanced systems involving light of wavelength <700nm (photosystem I) and of <680nm (photosystem II).



Scheme 1.1.2: Photophosphorylation

As shown in scheme 1.1.2 photosystem I produces a strong reductant and weak oxidant, whereas the converse case arises from photosystem II. The interaction of the weak oxidant with the weak reductant ultimately leads to ATP (4) synthesis. This process is termed photophosphorylation.[7]

In chemotrophic species ATP (4) is produced as a result of a series of degadative processes which can be classified into three stages:

a) breakdown of macro-molecules into smaller compounds,
b) degradation of these small compounds into the basic units of life,
c) complete combustion of these units to carbon dioxide.

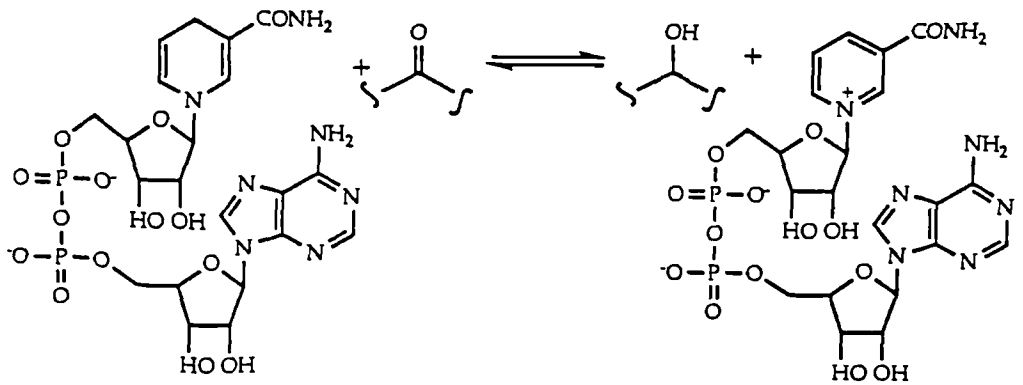
Nearly all of the ATP (4) produced by this series of processes occurs in the final stage.

Since the studies to be detailed in this work involve a chemotrophic bacterium, *Pseudomonas fluorescens*, a brief overview of the degradation of foodstuffs follows.

1.1.3: Natural oxidants and reductants

Although many different reactions appear in the living world the scope of operations is limited in number. Of these reductions, oxidations, esterifications and hydrolyses account for a large fraction.

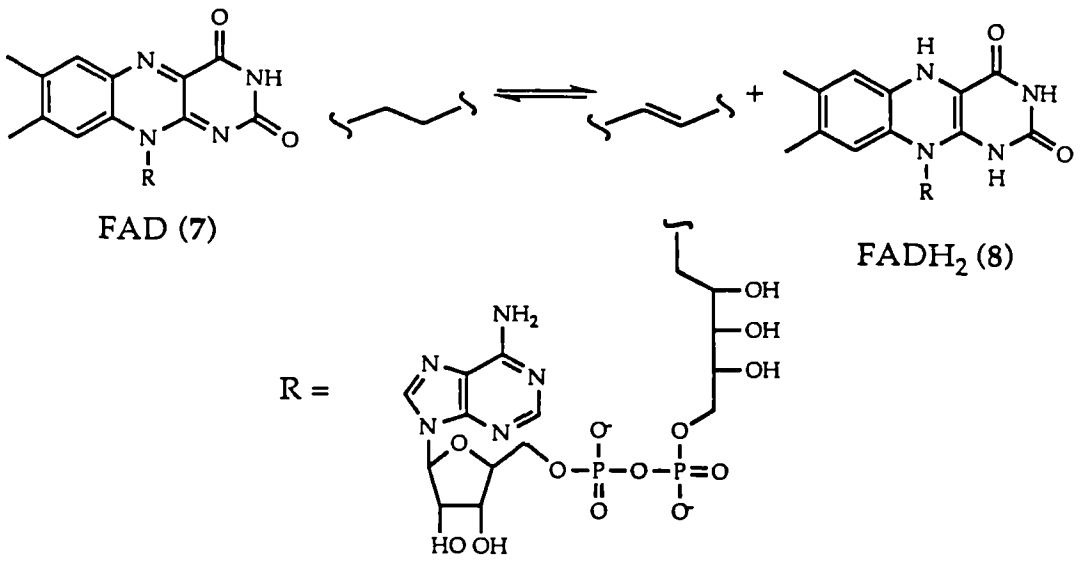
The reduction of ketones to hydroxyl functions is frequently mediated by nicotinamide adenine dinucleotide hydride (NADH, 5). This becomes nicotinamide adenine dinucleotide (NAD^+, 6) as a result, and this species carries out the reverse reaction, as shown in scheme 1.1.3.



NADH (5) NAD^+ (6)

Scheme 1.1.3: NADH (5)/NAD^+ (6) as reductant/oxidant.

For the dehydogenation of saturated carbon-carbon bonds, flavin adenine dinucleotide (FAD, 7) is frequently used. Again the end product, $FADH_2$ (8), is the operator for the reverse reaction, scheme 1.1.4.

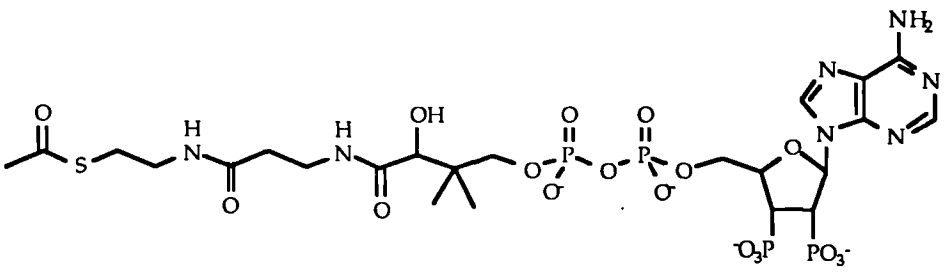


Scheme 1.1.4: FAD (7)/$FADH_2$ (8) as oxidant/reductant

The utilization of NAD^+ (5) leads to the formation of 3 molecules of ATP (4), and that of FAD (6) to the production of 2 molecules of ATP (4), as a result of an electron cycling system that ultimately induces a transfer of electrons to oxygen. This process is termed oxidative phosphorylation.[12]

Often, particularly in biosynthetic processes, FAD (7) or NADH (5) (and their counterparts) are phosphorylated. The abbreviations NADPH/$NADP^+$ and FADP/$FADPH_2$ are used in those cases.

As will be shown, acetate is a key entity for biological processes. Activation of this for biological purposes arises via the use of coenzyme A to produce acetyl CoA (9) .

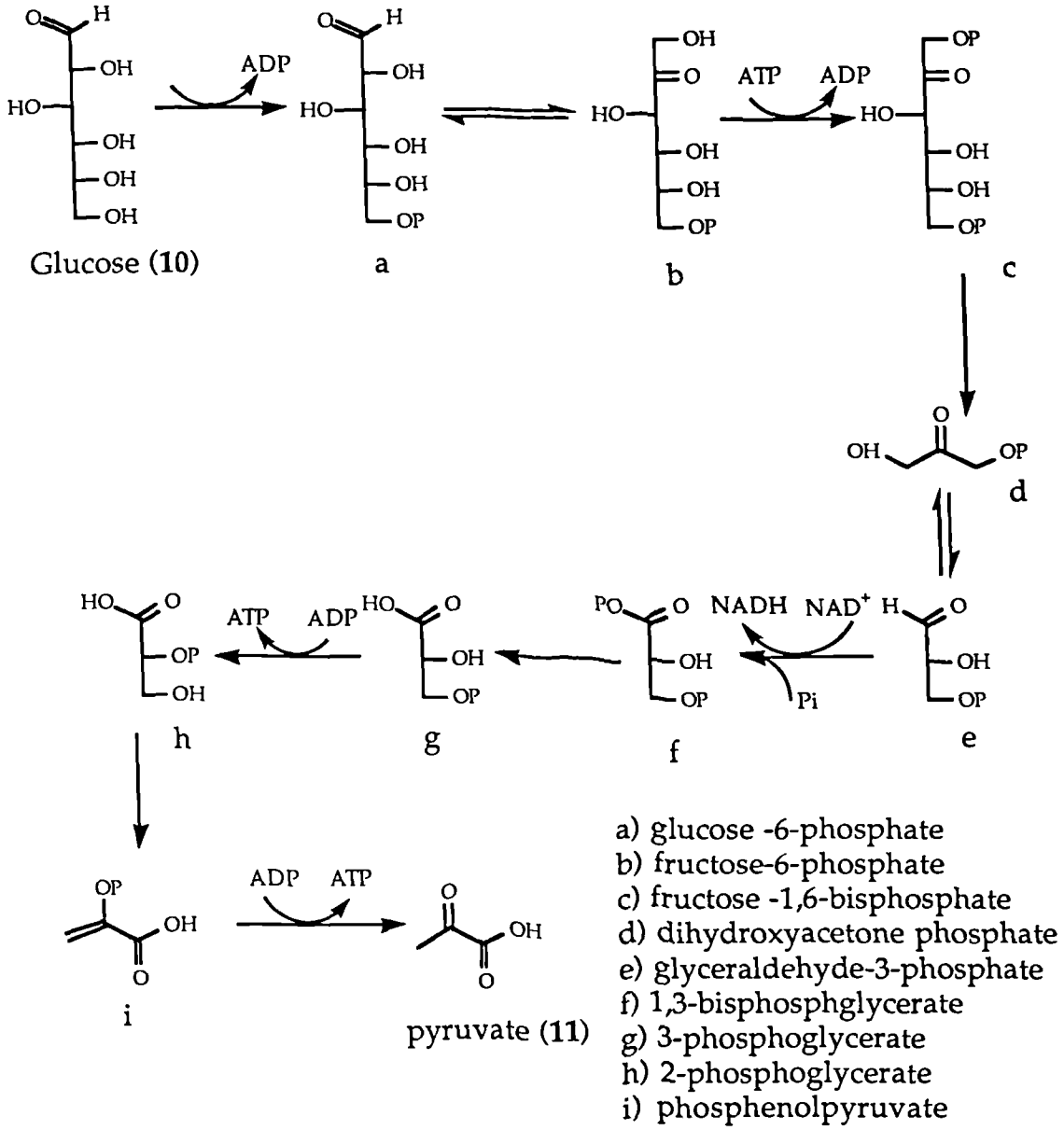


Acetyl CoA (9)

1.1.4: Primary chemotrophic metabolism

Throughout nature, primary metabolism has a dual role: the utilisation of foodstuffs as source of energy and simple building blocks of life, and the using this energy to build the components of life from these precursors.

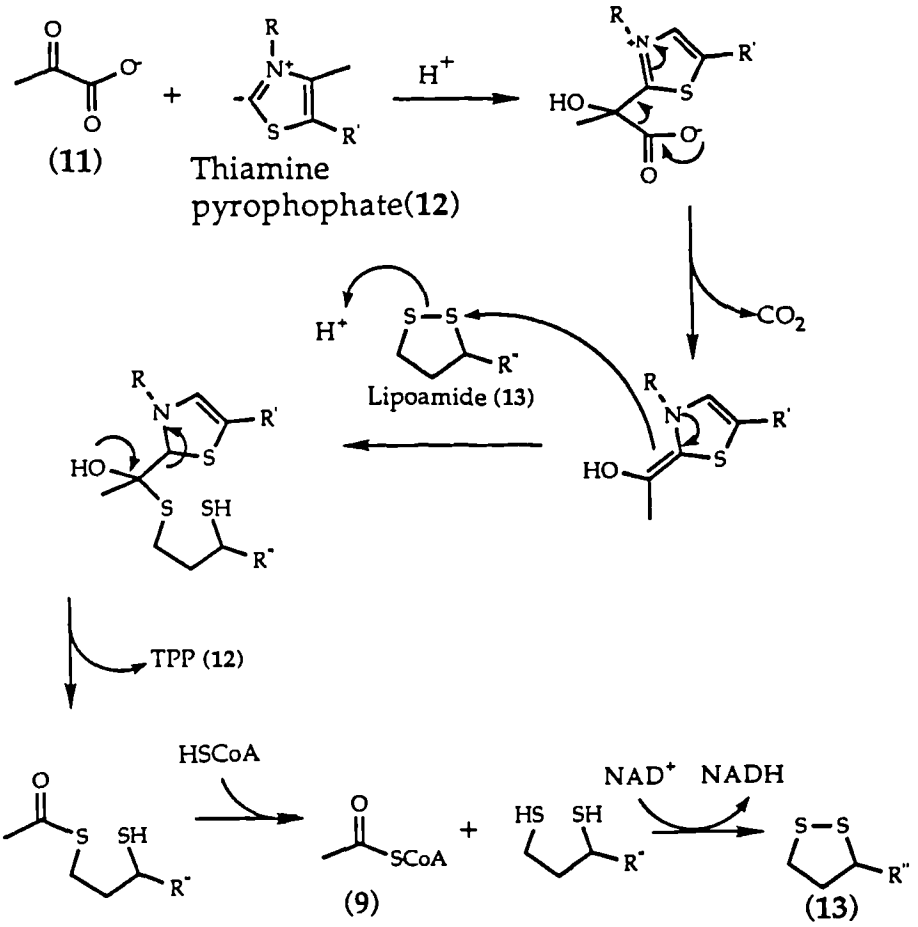
As previously mentioned one of the major sources of energy is carbohydrate, and in particular glucose (**10**). The glycolytic pathway is the ubiquitous process that degrades glucose (**10**) to pyruvate (**11**) as shown in scheme 1.1.5.



Scheme 1.1.5: Glycolysis

Although a little ATP (4) is generated overall by this process, it is by use of the Krebs' (or tri-carboxylic acid) cycle[9] that energy is most efficiently extracted via the complete combustion of acetate.[10]

Pyruvate is converted to acetyl CoA (9) as shown in scheme 1.1.6, with thiamine pyrophosphate (12), lipoamide (13) and NAD^+ (6) serving as co-factors.

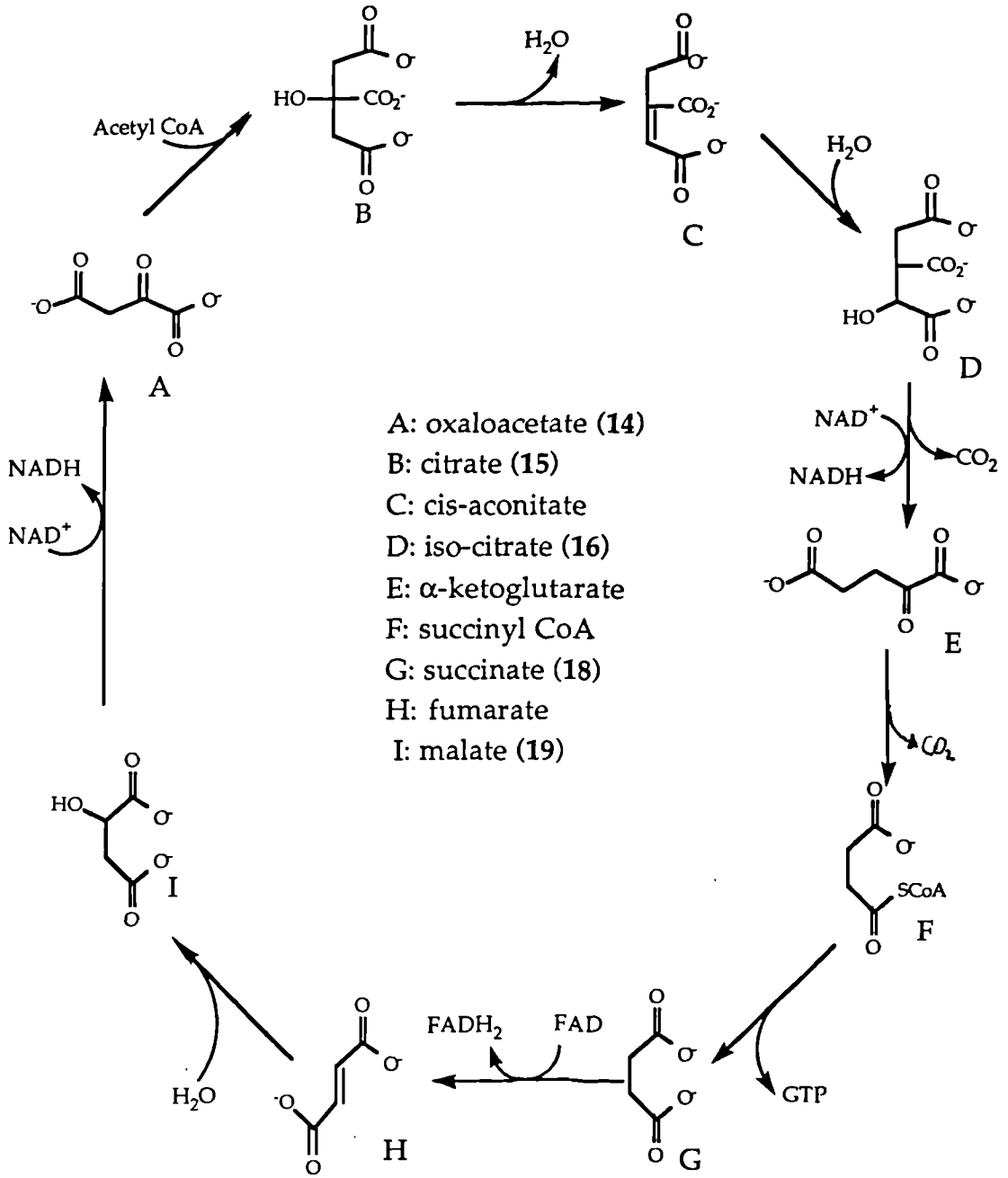


Scheme 1.1.6: Conversion of pyruvate (11) to acetyl CoA (9)

Acetyl CoA (9) then enters the Krebs' cycle via condensation with oxaloacetate (14) to produce citrate (15) as shown in scheme 1.1.7. A series of reactions is then under taken resulting in complete combustion of acetyl CoA (9) and regeneration of oxaloacetate (14).The potential net gain of ATP (4) from this series of reactions, and through other electron cycling systems, is 38 molecules per molecule of glucose (10).

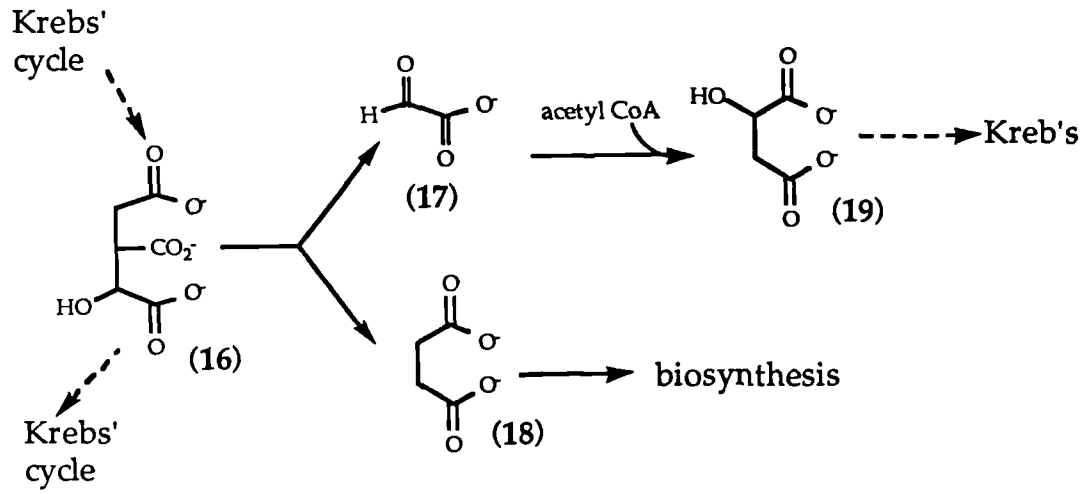
Simple organisms have a secondary part to this cycle, termed the glyoxylate pathway. The regulatory step occurs at isocitrate (16) (scheme 1.1.8), which can be alternatively lysed to yield glyoxylate (17) and succinate

(18). The succinate (18) is available for biosynthetic purposes, whereas glyoxylate re-enters the Kreb's cycle via condensation with acetate to yield malate (19).



Scheme 1.1.7: The Krebs' cycle

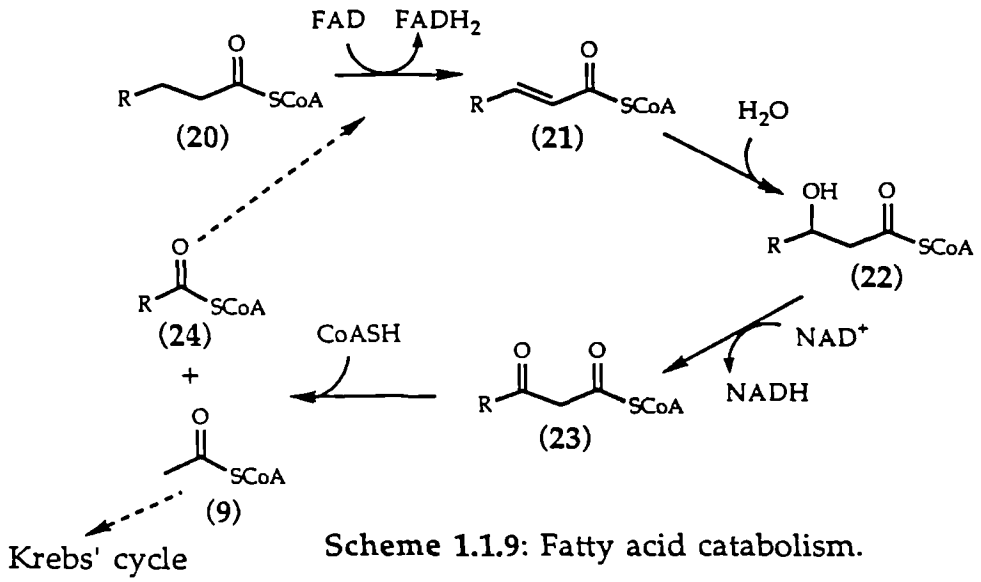
In using two molecules of acetate and producing fewer molecules of ATP (4), this cycle has a reduced energy producing efficiency. However in times of high food-stuff availability the succinate produced can now be utilised entirely for biosynthetic purposes.



Scheme 1.1.8: The glyoxylate cycle

1.1.5: Energy storage

For cellular processes ATP (4) is a transitory form of energy, used up rapidly after production. For long term energy storage glycogen, a polymer of D-glucose (10), and fatty acids are synthesised. These can then be readily degraded (catabolised) in order to retrieve the energy contained within. Glycogen, after degradation to glucose (10) which undergoes gylcolysis to yield ATP (4). However fatty acids are degraded by a different process, termed β-oxidation,[11] as shown in scheme 1.1.9

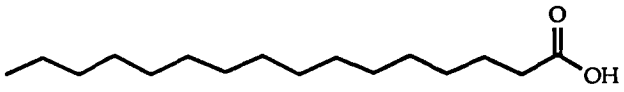


Scheme 1.1.9: Fatty acid catabolism.

Acyl CoA fatty acid derivatives (20) undergo degradation via a series of steps that formally resemble the last few stages of the Krebs' cycle leading

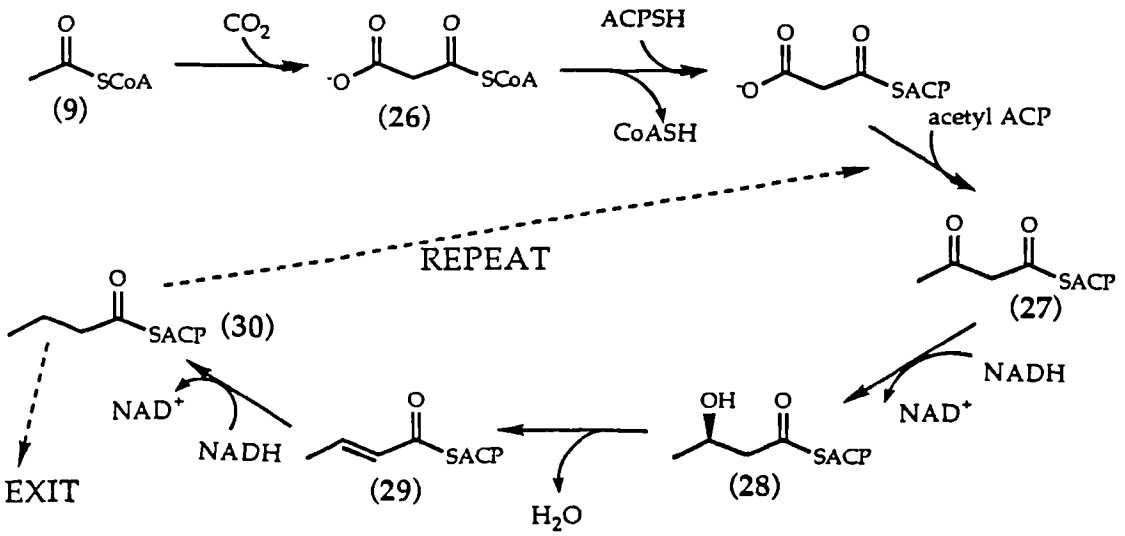
to oxaloacetate regeneration (**14**). Thus dehydrogenation to yield the α,β unsaturated thioester (**21**) is followed by hydration to produce a 3-hydroxythioester (**22**). Oxidation to the β-keto derivative (**23**), prior to thiolysis with coenzyme A forms one molecule of acetyl CoA (**9**) and a new acyl CoA (**24**). This acyl CoA (**24**) can under go the same cycle of reactions whereas the acetyl CoA (**9**) is now available either for biosynthetic procedures or combustion within the Krebs' cycle.

As a result of this process palmitic acid (**25**), for example, can be completely combusted, producing 129 molecules of ATP (**4**) as a result.



Palmitic acid (**25**)

Fatty acids are biosynthesised for later retrieval by the process shown in scheme 1.1.10. Acetyl CoA (**9**) is carboxylated to malonyl CoA (**26**), which is then transferred to an acyl carrier protein (ACP). Condensation of this with acetyl ACP yields acetoacetate ACP (**27**) with concomitant release of carbon dioxide. Reduction by NADPH produces (3R)-3-hydroxybutanoyl ACP (**28**), which then under goes loss of water to produce (2E)-but-3-eneoyl ACP (**29**). A final hydrogenation step yields the fully saturated butanoyl ACP (**30**) which can then under go further homologation until the desired chain length has been achieved, at which point the cycle is terminated.



Scheme 1.1.10: The biogenesis of fatty acids.

Fatty acid metabolism is essentially identical in all living systems. However the enzymes involved show a range of structural differences.[11,12] Although they all possess the required catalytic domains, namely β-ketoacyl synthase, acetyl transferase, β-hydroxyacyl dehydratase, malonyl transacylase, enoyl reductase, β-ketoacyl reductase, thioesterase and an acyl carrier protein, the arrangement of these can vary.

Two classes of fatty acid synthase have been identified:

TypeI: Of mammalian origin, these exist as single multi-functional peptides with a globular arrangement of catalytic domains and a molecular weight of appproximately 5×10^6. A further sub-division of this type of fatty acid synthase are found in yeasts and some fungii, whereby two sub-units co-operate. The α-subunit includes the β-ketoacyl reductase, β-ketoacyl synthase and the acyl carrier protein. The β-subunit contains the remaining activities. In active form the enzyme is $\alpha_6\beta_6$ complex of molecular weight 2.4×10^6.

Type II: Found in plants and most bacteria, these exist as eight structurally independant mono-functional enzymes.

Fatty acid biosynthesis has been intensely studied,[12] and the elucidation of the stereochemical aspects of the process serves as an excellent example of the use of isotopic labelling studies. This will be described in the next section, which discusses the methodology used in the investigation of biogenetic processes.

1.2: BIOSYNTHETIC METHODOLOGY.

1.2.1: Isotopic labelling.

The biologically common elements all exist predominantly in one isotopic form, but small amounts of other isotopes are available. Thus if a compound containing an 'unnatural' isotope is used instead of the more common form it may be possible to determine whether the isotope, and therefore the compound, is incorporated into the required metabolite.

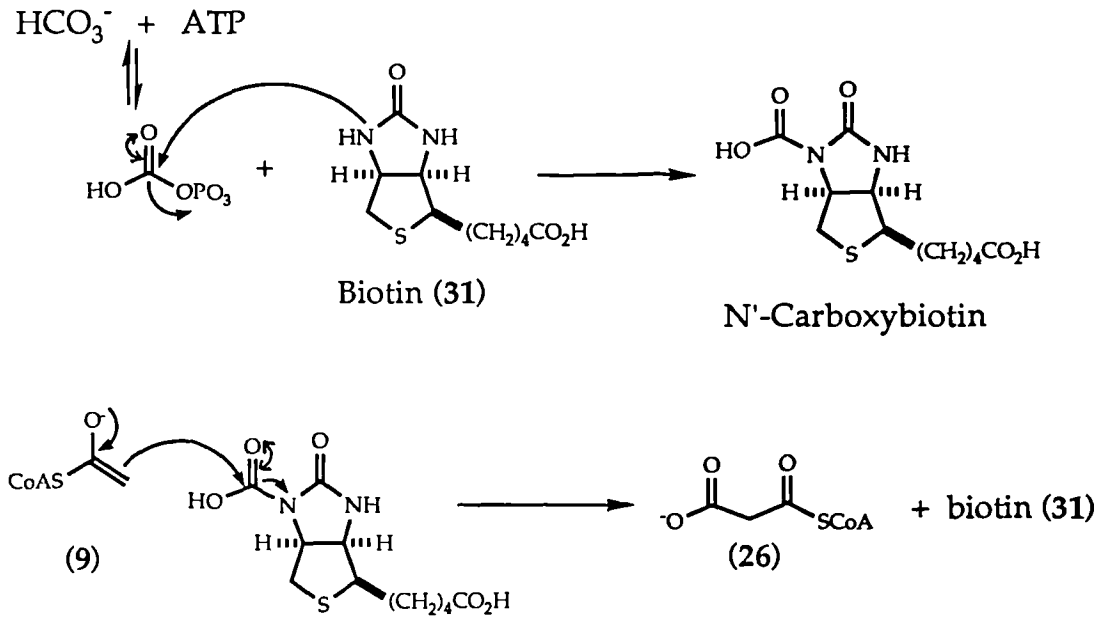
Two types of isotope may be used, stable or radioactive. For classical biosynthetic studies, radio-labels were chosen owing to the relative ease of

detection. The introduction of readily available powerful spectroscopic methods within the last twenty years, however, has facilitated the use of stable labels such that those studies have become the more common type.

1.2.2: The elucidation of fatty acid biosynthesis using radio-labels.

That acetate was a precursor to the fatty acids was initially demonstrated in 1945 with the use of stable labels.[13] The fatty acids isolated from rats after administration of [1-^{13}C, 2-$^{2}H_3$]-acetate were completely combusted. The water resulting from this process was isolated and comparison of the refractive index to that of $^{1}H_2O$ showed the label to have been incorporated. Mass spectral analysis of the CO_2 produced showed that the ^{13}C label had also been incorporated.

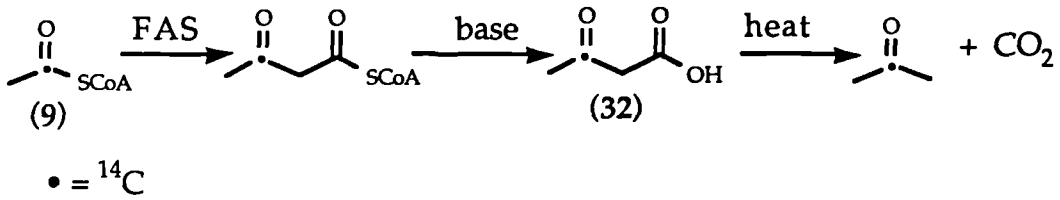
Later work using $H^{14}CO_3$ showed that CO_2 was required for the fatty acid biogenetic process, but was not contained within the final product.[14] This was explained by showing that malonate, as opposed to acetate, was the effective chain extension unit with the carbon dioxide being used to carboxylate acetyl CoA (9) and then being released upon subsequent condensation with a second molecule of acetyl CoA (9). The carbon dioxide fixing operation was shown to be mediated by biotin (31),[15] scheme 1.2.1.



Scheme 1.2.1: The fixing of carbon dioxide mediated by biotin

A purified fatty acid synthase (FAS) enzyme derived from baker's yeast was later incubated with [1-^{14}C] acetyl CoA (9), and the process terminated via protein precipitation. Base hydrolysis led to the release of an enzyme

bound intermediate which was shown to be acetoacetate (32). Decarboxylation of this and isolation of the acetone produced showed the label to have been incorporated into C-3 of acetoacetate (32), scheme 1.2.2.

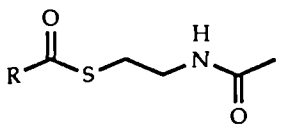


Scheme 1.2.2: Incorporation of label from acetate (9) into acetoacetate (32)

When acetoacetyl CoA (27) was incubated with the enzyme in the presence of NADPH (5), production of (3R)-3-hydroxybutanoyl CoA (28) was observed. Incubation of this with the enzyme led to production of *trans*-but-2-enoyl CoA (29). The (3S) isomer did not give rise to any UV active products, showing it not to be accepted as a substrate.[15,16]

Only the *trans*-but-2-enoyl CoA (29) was accepted by the enzyme to form the fully saturated butanoyl CoA (30), the *cis*- isomer being found not to be a substrate.

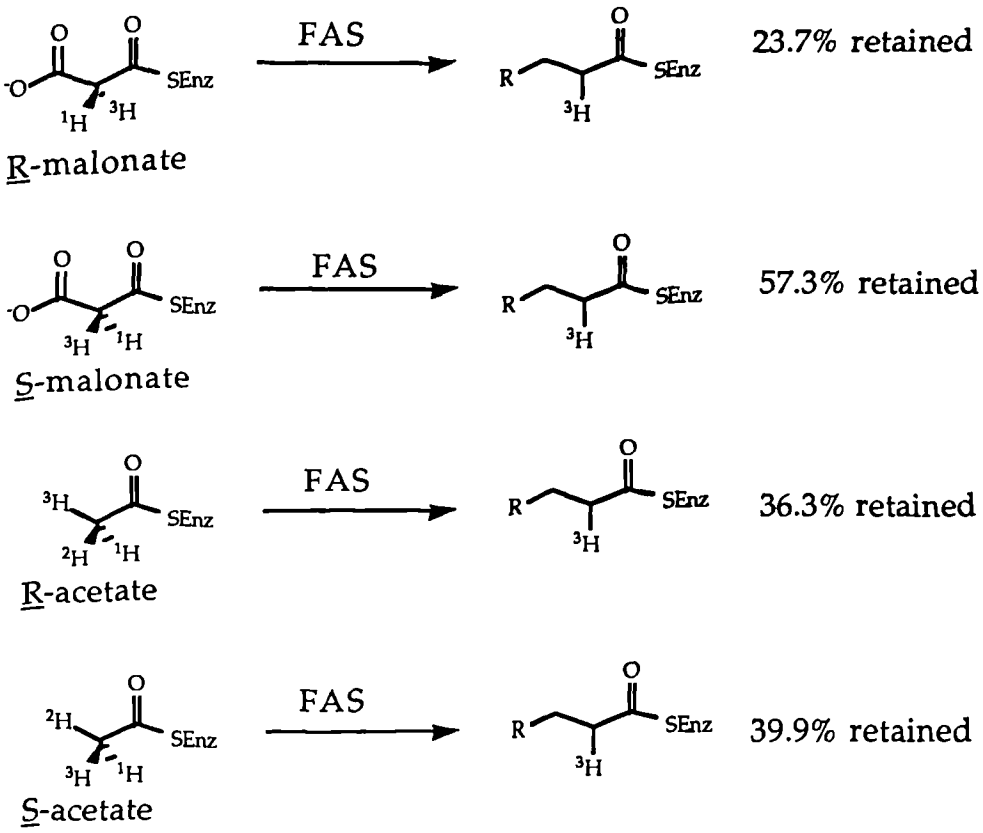
It is note-worthy that these experiments were also carried out using the substrates in the form of their N-acetylcysteamine thioesters (33). This thiol contains the same structure as the terminus of the pantotheinic acid side chain of coenzyme A (see acetyl CoA (9)). It was hoped that such substrates would be accepted by the enzyme in a manner analagous to the CoA forms. This was indeed found to be so.



A general N-acetylcysteamine thioester (33)

In monothioester-monoacid form, malonate is prochiral. The stereo specific introduction of a ^{3}H label into malonate meant that the 2R and 2S forms could be incubated with the enzyme to determine any stereospecificity in the biogenesis of fatty acids. The utilisation of all three isotopes of hydrogen meant that chiral acetates could be similarly employed.

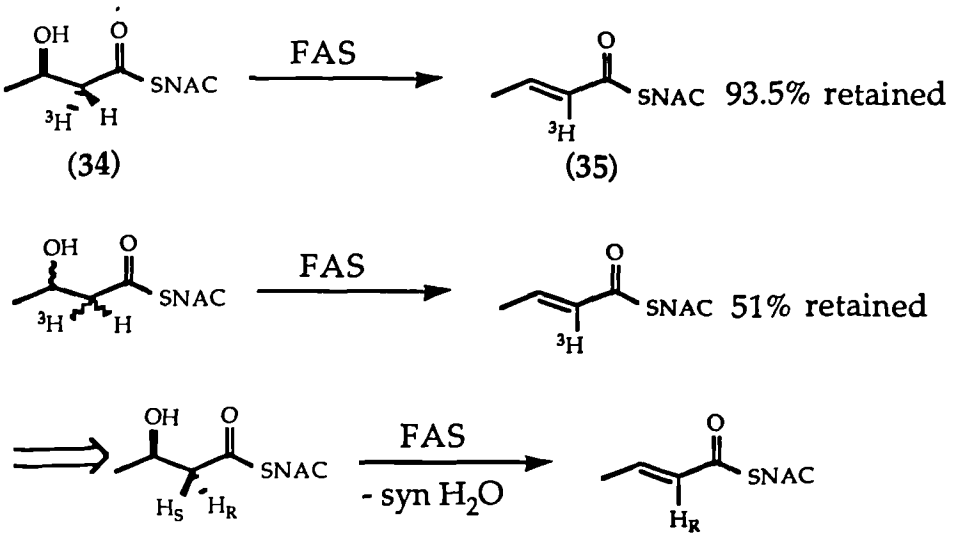
Such experiments, as shown in scheme 1.2.3, indicated that the conversion of acetyl CoA into malonyl CoA proceeded retentively with the overall conversion of acetate to fatty acid being stereospecific.[17,18]



Scheme 1.2.3: The use of chiral acetate and malonate in the elucidation of fatty acid biosynthesis

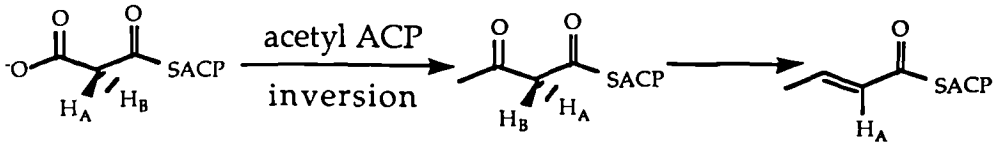
The use of (2R)- and (2S)-[2-$^2H_1$,$^3H_1$]-malonates gave rise to preferential uptake of the ^3H label from the (2S)-enantiomer.[18] The condensation step leading to acetoacetate (**32**) could proceed with either retention or inversion of configuration at C-2 of malonate. As previously described, the reduction of acetoacetate leads to specifically the (3R)-isomer (**28**), with further dehydration leading to the *trans* isomer (**29**). Therefore an experiment was required to determine the stereochemical course of the dehydration, as this would also yield information regarding the condensation step.

(2R, 3R)-[2-^3H]-3-hydroxybutanoic acid (**34**) was synthesised, converted to the N-acetylcysteamine thioester and then incubated with the enzyme. Upon isolation of the *trans*-but-2-eneoate (**35**), 93.5% of the label was found to be retained. A control experiment with non-stereospecifically labelled [2-$^3H_1$]-3-hydoxybutanoate led to the loss of approximately 50% of the label. This demonstrated that the (pro-2S) hydrogen was removed, indicating loss of water in a *syn* manner as shown in scheme 1.2.4.



Scheme 1.2.4: The loss of the pro-2S hydrogen during the dehydration step in fatty acid biosynthesis

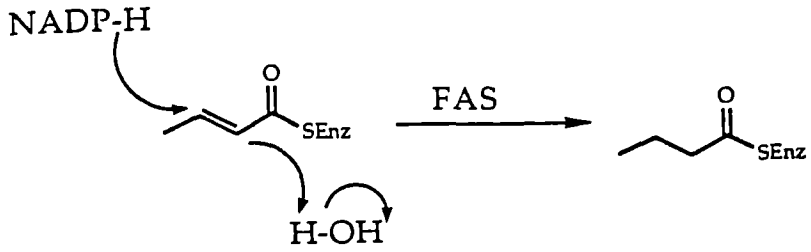
Since it was known that the label from the 2S enantiomer of malonate was incorporated into the final metabolite, but that the pro-2S hydrogen had now been shown to be specifically eliminated during dehydration, the deduction that the condensation of acetyl ACP with malonyl ACP occurs with inversion of configuration at C-2 of malonate, as shown in scheme 1.2.5, was made.



Scheme 1.2.5: inversion of configuration at C-2 of malonylACP during the condensation with acetyl ACP.

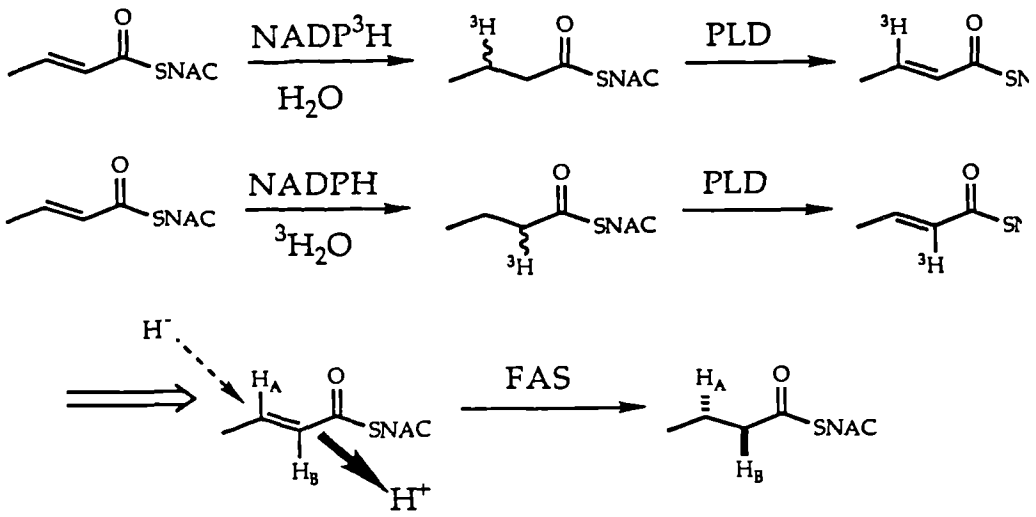
This only left the saturation step to be investigated.Previous work had shown that the hydride introduced to C-3 was NADPH (5) derived,[19] whereas the proton at C-2 came from the aqueous medium,[20] as shown in scheme 1.2.6.

Further work involved incubating the N-acetylcysteamine thioester of *trans*-but-2-enoate with the enzyme in the presence of either $NADP^3H/H_2O$, or $NADPH/^3H_2O$.



Scheme 1.2.6: The origin of the hydrogens during the final reductive step of fatty biosynthesis

Dehydrogenation of the resultant fatty acid with pig liver dehydrogenase (PLD), an enzyme which was known to specifically remove the pro-2R,pro-3R hydrogens, was carried out.



Scheme 1.2.7: The anti delivery of hydrogen to the si faces in the final reductive step of fatty acid biosynthesis.

It was found that label from NADP^{3}H (**5**) was retained after this operation, as was that from ^3H_2O. Thus it had been demonstrated that the final reductive step proceeds with anti delivery of hydrogen to the si faces of the prochiral alkene, scheme 1.2.7.

Subsequent studies have shown that this final enoyl reduction step varies across species.[21,22]

These investigations demonstrated how the use of isotopic labelling, biomimetic compounds, purified enzymes and outstanding ingenuity could lead to the unravelling of the stereochemical course of biosynthetic processes.

The use of radio labels has a major disadvantage, however. Determination of the position of label incorporation within a metabolite requires careful and tedious degradative work, resulting in loss of often valuable material. Prolonged exposure to radio-activity is also undesirable. For studies of these kind, the introduction of high power nmr techniques has led to an increased use of stable labels for biosynthetic investigations.[23]

1.2.3: The use of stable isotopic labels.

The use of nuclear magnetic resonance spectroscopy has greatly enhanced progress in many areas of chemical research. In particular structural determinations of unknown compounds, especially natural products, can now be achieved without the need to degrade material. Additionally, very little compound is required for such experiments.

For many years ^{1}H was the only experimentally useful isotope. The introduction of readily accessible Fourier Transform nmr, however, has led to a proliferation in the isotopes which can be observed, such that the availability of an isotope is often the limiting factor in the design of experiments.

For biosynthetic investigations specific incorporation of labels can be determined with relative ease providing the complete spectral properties of the natural product are known. Indeed it is the full assignment of resonances in the nmr spectrum which often proves to be the trickiest part of such studies.

The isotopes commonly used in conjunction with such investigations are ^{1}H, ^{2}H and ^{13}C. Of these ^{13}C is often the most productive. The isotope accounts for only 1.1% of all naturally occurring carbon, with the major isotope being nmr inactive. Chemical synthesis can be used to introduce high enrichment of this isotope within a putative biosynthetic precursor. This is important, as administered compounds will frequently be directed along more than one metabolic pathway, leading to large dilutions of the applied label.

The chemo-magnetic environments of carbon atoms lead to large variations in the relaxation time of the nucleus after initial excitation. This results in variations in the intensity of spectral signals, that are unrelated to the relative amounts of carbon present. Thus, to ensure that any observed enhancement is 'real,' a high ^{13}C content in the administered compound is desirable.

In itself the use of a single ^{13}C label does not lead to any increase in the amount of information to be gained with respect to the use of radio-label. Any advantages arise from the analysis being less tedious and material needing not to be destroyed.

Further information may be gained through spectroscopic methods, however, by the utilisation of multiple labelling techniques, either homo- or heteronuclear. Thus if two adjacent carbons in a molecule are labelled with ^{13}C it is effectively the bond that is labelled. Any incorporation of these atoms as a unit (ie bond incorporation) will be observed via ^{13}C-^{13}C couplings in the nmr spectrum. The low natural abundance of ^{13}C means that such couplings are not observed under normal conditions.

A heteronuclear bond, for example C-O, can be labelled by the use of ^{13}C and ^{18}O. Although ^{18}O is nmr inactive the different nuclear properties of this with respect to ^{16}O mean the carbon resonance has a slightly different value. This shift is usually visible with only a little extra analytical work being required. Similar possibilities exist for the use of ^{13}C-^{2}H and ^{13}C-^{15}N labelling.

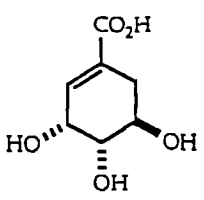
Such techniques have been readily applied to the investigations of secondary metabolic processes, as will be demonstrated in the next section.

1.3: SECONDARY METABOLISM.[24]

1.3.1: Introduction

Secondary metabolism was previously described as being non-essential to life and consisting of metabolic processes restricted to a few species. Whilst this is true in terms of the metabolites expressed by such pathways, the types of processes themselves are readily classified and exist in many species.

Thus metabolites derived from shikimic acid (36), mevalonic acid (37) and amino acids are found throughout nature. However, the resultant structures of the metabolites can show tremendous variations.



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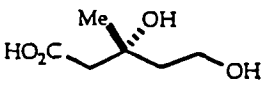
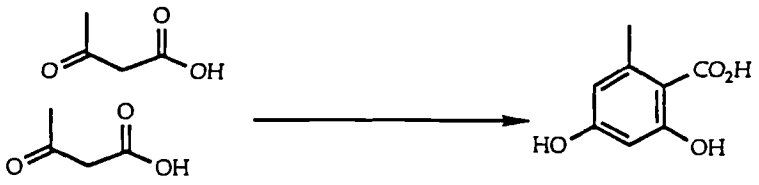


(37).

Perhaps the most striking examples of this are found in metabolites resulting from the polyketide pathway.

1.3.2: The polyketide biosynthetic pathway.

At the dawn of the present century a series of biosynthetic hypotheses were forwarded by Collie, one of which described the biogenesis of orsellinic acid (38) from two molecules of acetoacetate (32), scheme 1.3.1.[25]

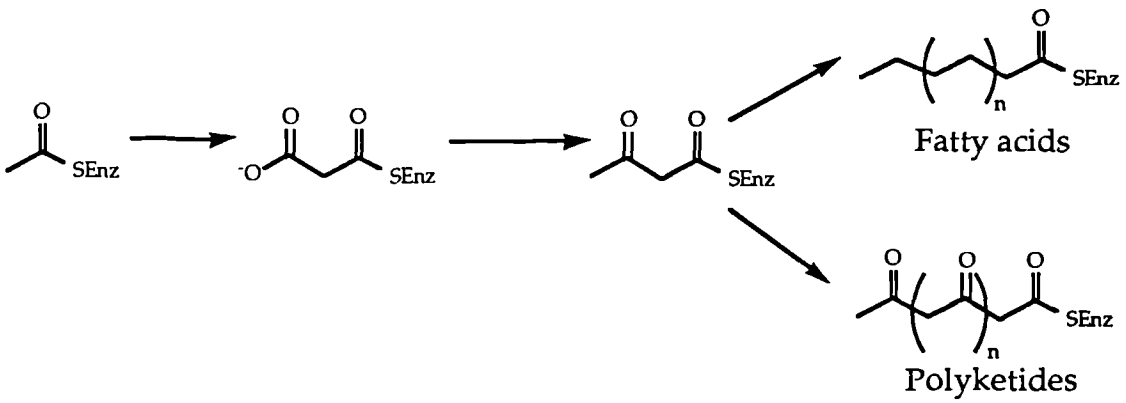


(32) (38)

Scheme 1.3.1: The biosynthesis of orsellinic acid (38) as proposed by Collie.

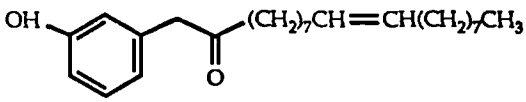
Such ideas were largely ignored until after the Second World War and discovery of the involvement of acetyl CoA (9) in fatty acid biogenesis. Resurrection of Collie's ideas by Birch arose from a hypothesis invoking the retention of β-keto functions in the linear chains resulting from sequential acetate-malonate condensations, as shown in scheme 1.3.2.[26]

Subsequent modifications of the the polyketide chains, by Claisen ester or aldol type reactions and aromatisation, could then lead to the observed natural products.



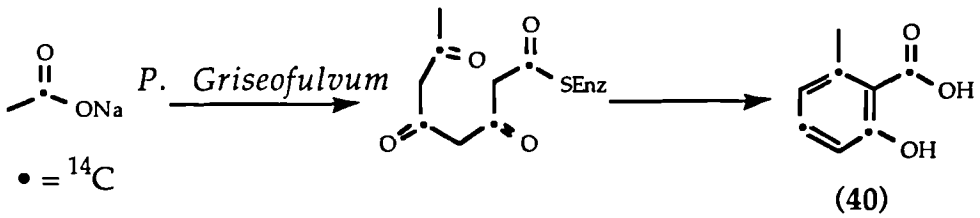
Scheme 1.3.2: Acetate-malonate condensations leading to fatty acids or polyketides

The first natural product recognised as being derived from such a process was campnospermonol (39).[27]



(39)

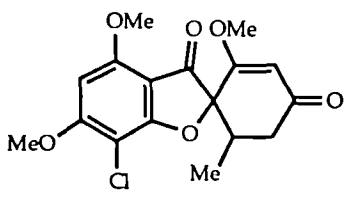
Experimental evidence was first obtained from the utilisation of [1-^{14}C] acetate, which was administered to the fungus *Penicillium griseofulvum*. Amongst a range of metabolites produced by this species is 6-methylsalicylic acid (6-MSA, 40). Degradation of this after isolation led to the determination of the sites of label incorporation, and confirmation of the hypothesis, scheme 1.3.3.[28]



(40)

Scheme1.3.3: The biosynthesis of 6-methylsalicylic acid (40)

Further experiments showed that other metabolites were of polyketide origin, including griseofulvin (41)[29] and curvularin (42).[30] The original natural product chosen by Collie, orsellinic acid (38), was also shown to be the result of this pathway. Confirmation that the oxygen atoms in this metabolite were from 'unreduced' acetate was achieved from the use of [1-^{14}C, ^{18}O] acetate.[31]



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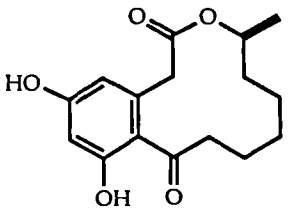
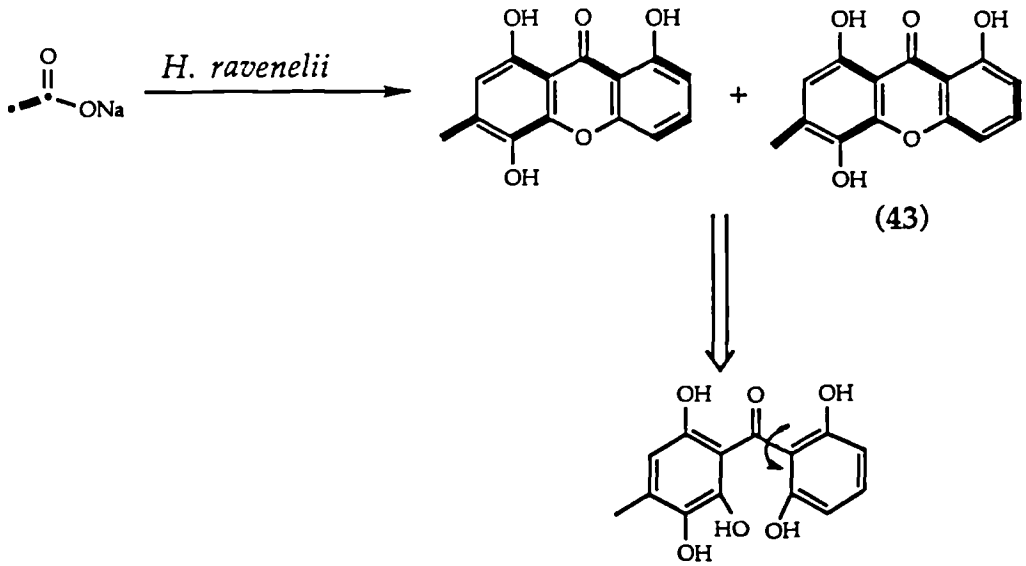


(42)

That polyketide biosynthesis was analgous to fatty acid biosynthesis with respect to an acetyl starter unit condensing with a malonyl chain extender unit was also demonstrated.[32]

The introduction of high field FT nmr capabilities led to the possibility of gaining further insights into this biosynthetic process, particularly with respect to the poly-β-keto chain configuration that leads to the final metabolite. This was demonstrated by studies regarding the biosynthetic origin of ravenelin (43).[33]

Thus the administration of [1,2-$^{13}C_2$]-acetate to cultures of *Helminthosporium ravenelii* resulted in ^{13}C-^{13}C coupling in the ^{13}C nmr spectrum, as shown in scheme 1.3.4. It was shown, therefore, that a symmetrical precursor was an intermediate on the biosynthetic pathway, and that this led to randomisation of label.



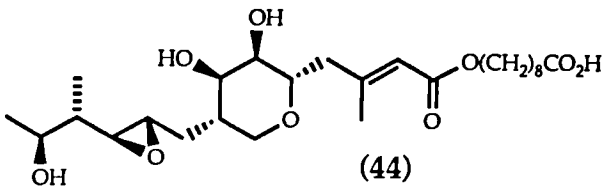
Scheme 1.3.4: The biosynthesis of ravenelin (43)

Such techniques have been used on a variety of metabolites and have proven to be highly productive.[23]

The pioneering work of Birch has therefore been expanded to show that polyketide biosynthesis is a widely occurring metabolic phenomenon, analgous to fatty acid biogenesis but lacking in the reduction, dehydration, and reduction steps. This pathway has been adopted in many different species, including bacteria, fungi and some plants, to produce metabolites of astonishing multifariousness.

Extensive research into the biosynthesis of polyketide metabolites has been undertaken since the pioneering work of Birch. As a result of this work the 'classical' polyketide hypothesis having been supplanted over the last decade. New hypotheses regarding the biogenetic origins of

polyketide natural products are discussed in depth within chapter three, with relevance to the biogenetic origin of pseudomonic acid (44).



(44)

Aspects regarding the discovery and utility of this natural product, and studies directed towards the elucidation of its biosynthesis, are presented in chapter two.

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CHAPTER TWO

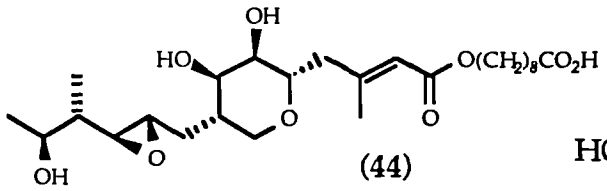
PSEUDOMONIC ACID

2.1 : THE ANTIBIOTIC PSEUDOMONIC ACID

2.1.1 :Discovery of the antibiotic

The growth inhibitory effects of *Pseudomonas fluorescens*, a Gram negative bacterium[1] of the *Pseudomonadaceæ* family[2], upon various fungi, bacteria and yeasts were first noted in 1887.[3] Further reports on the biological activity of cultures and culture extracts were made over the ensuing decades[4]. No chemical entity was identified to account for this behaviour, however, until 1971 when a purified acidic extract was demonstrated to exhibit nearly all the observed activity.[5]

This compound was termed pseudomonic acid (**44**) and structural elucidation studies showed it to possess a unique carbon framework,[6] including a 1,2-dihydroxy substituted tetrahydropyran ring, an epoxide functionality, an α,β unsaturated ester, with the condensed alcohol being the unprecedented 9-hydroxynonanoic acid (**45**). Further work showed the absolute stereochemistry to be as depicted in figure 2.1.1.[7]



(**44**)

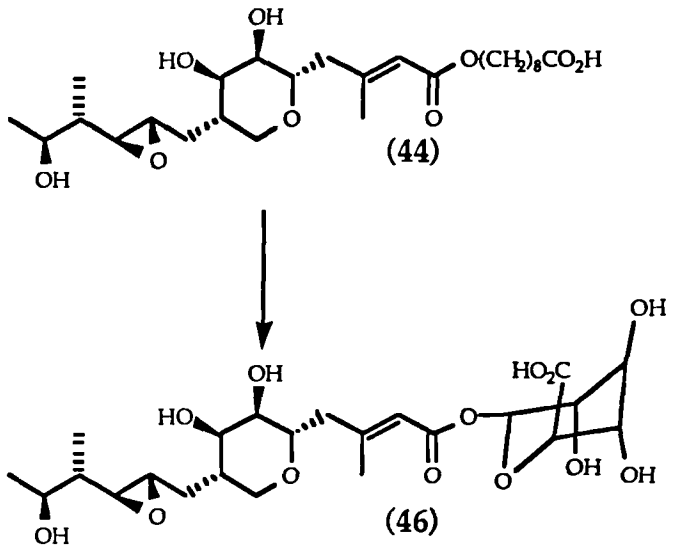
$HO(CH_2)_8CO_2H$

(**45**)

Figure 2.1.1: The absolute configuration of pseudomonic acid (**44**)

The biological activity of this agent was found to be restricted to the pH range 4 to 9,[5] but within these limits it showed high levels of activity against Gram positive bacteria, especially *Staphylococci* and *Streptococci* species,[8] although only a limited number of Gram negative species were affected. Interestingly, it exhibited no cross-resistance with other clinically available antibiotics. Binding to human serum was found to be high (95%) but toxicity levels were low. Subcutaneous administration to mammals resulted in rapid excretion, via hydrolysis, of mainly monic acid β-glucoronide (**46**),[9] as shown in scheme 2.1.1.

As a result clinical applications of the metabolite are restricted to the topical treatment of skin infections.[10] Currently it is marketed by SmithKline Beecham under the generic name Mupirocin as the active component (2%) of the skin cream Bactroban.



Scheme 2.1.1: Excretion of monic acid β-glucoronide (46) after pseudomonic acid (44) adminstration

2.1.2: The mode of operation of pseudomonic acid.

Early studies on the modus operandii of the antibiotic showed it to inhibit protein biosynthesis[11] which was later found to be the result of competitive inhibition of isoleucyl t-RNA synthetase.[12] It has been suggested that the epoxy side-chain is the moiety responsible for such activity, as shown in figure 2.1.2. This side chain has the same carbon backbone of isoleucine (47), and so may compete for the binding sites on the enzyme.[12]

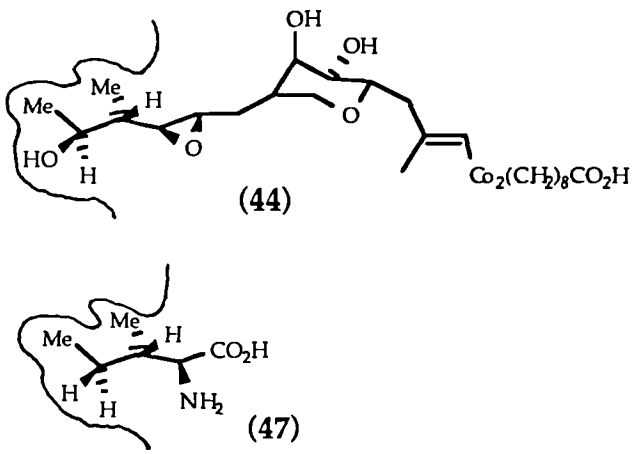
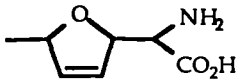


Figure 2.1.2: The structural similarity between pseudomonic acid (44) and isoleucine (47), with respect to isoleucyl t-RNA synthase

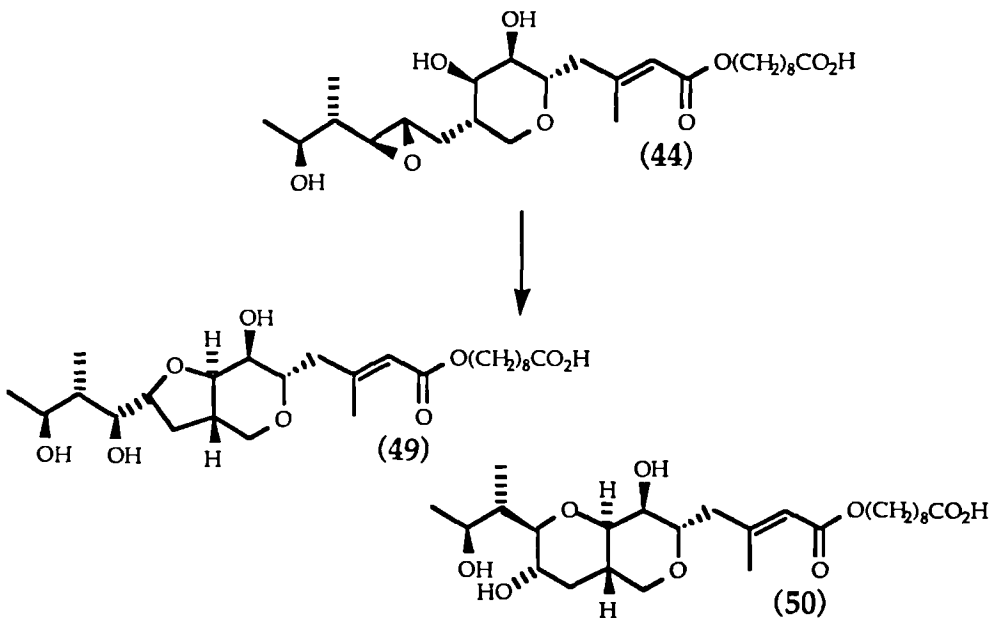
Resistance to the antibiotic by the producing organism was shown to be through a modified isoleucyl t-RNA synthetase. This was found to have an affinity for isoleucine (47) over pseudomonic acid (44) of 6 orders of magnitude greater than other prokaryotic isoleucyl t-RNA synthetases.[13]

Only one other natural product, furanomycin (48),[14] has been shown to exhibit comparable behaviour,[15] but this has found no clinical utility. In conjunction with the unique structure of pseudomonic acid (44), this explains the lack of cross-resistance of the antibiotic against other clinically available antibiotics.



(48)

As mentioned above, the biological activity is only observed within pH limits of 4 to 9.[5] This results from the instability of pseudomonic acid (44) to strong base or acid.[16] At low pH, rapid rearrangement (30 minutes) occurs, leading to a 1.6:1 mixture of the isomeric compounds (49) and (50), scheme 2.1.2. Under strongly alkaline conditions, a slower rearrangement (18 hours) takes place, yielding the same products but in a ratio of 3.7:1. The fact that it is the epoxy function which is disrupted as a result of this supports the theory that this part of the antibiotic is responsible for the observed biological activity.



Scheme 2.1.2: Rearrangement of pseudomonic acid in acid or base.

2.1.3: Other pseudomonic acid metabolites

Further isolation work on extracts from *Pseudomonas fluorescens* cultures gave rise to the discovery of 3 further members of the pseudomonic acid family. These are named pseudomonic acid B (**51**),[17] C (**52**),[18] and D (**53**),[19] with the parent now termed pseudomonic acid A (**44**). Recent work at SmithKline Beecham has resulted in the extraction of a fifth metabolite from cultural broths, pseudomonic acid E (**54**),[20] as shown in figure 2.1.3.

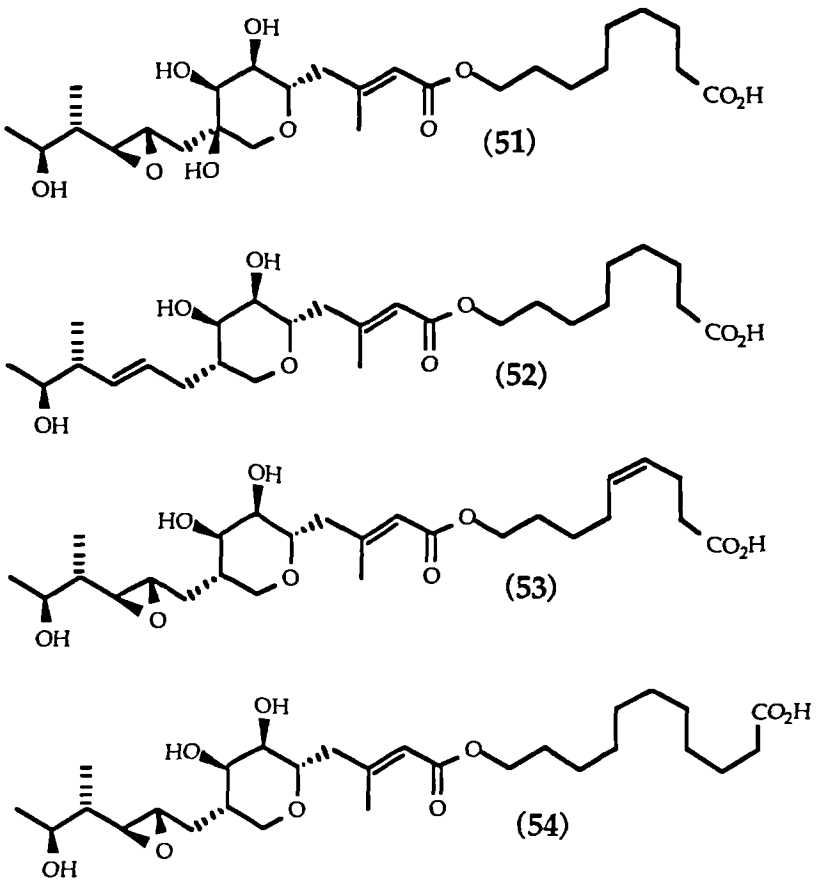


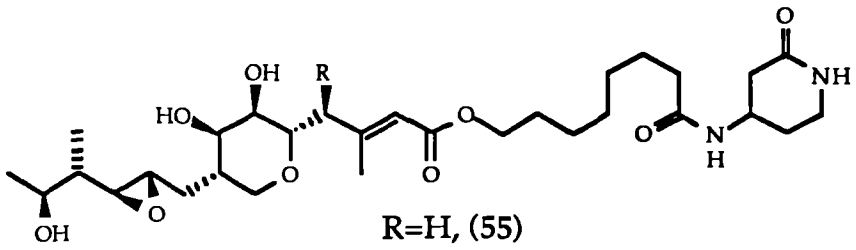
Figure 2.1.3: Co-metabolites of pseudomonic acid (**44**)

These minor metabolites have biological activities similar to or less than that of pseudomonic acid A. Within this work, the term pseudomonic acid will refer to the major metabolite. (**44**).

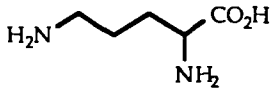
The discovery of two pseudomonic acid derivatives (**55**) and (**56**), isolated from an unidentified pink bacterium, itself isolated from the marine sponge *Darwinella rosacea*.[21] has been recently reported. In both these metabolites the 9-hydroxynonanoic acid chain has been replaced by an 8-hydoxyoctanamide side chain with what appears to be an amino δ-

lactam moiety derived from ornithine (57). The derivative (56) also possesses a 4-β-hydroxy function.

The biological activity of these metabolites is unknown, but their source leads to intriguing evolutionary questions.



R=H, (55)
R=OH, (56)



(57)

2.2 : PSEUDOMONIC ACID BIOSYNTHESIS

2.2.1 : Pseudomonic acid production by *Pseudomonas fluorescens.*

The strain of *Ps. fluorescens* originally selected for isolation studies was a soil isolate.[1,9] From this was developed a higher producing strain, NCIB 10586. This strain has been used for the majority of the studies reported herein.

The media used for growing *Ps. fluorescens* were based on those developed by workers at SmithKline Beecham.[22] Lyophilised samples of the bacterium were reconstituted prior to storage on nutrient deficient agar slopes. In this form the bacteria are viable for 4 months.

For use, a slope was flooded with nutrient broth in which the bacteria were resuspended. An aliquot of this was used to inoculate a primary medium, designed to promote efficient cell growth and replication. After 24 hours, a portion was transferred to a secondary medium in which metabolite production occurred.

The production was found to be complete within 48 hours of inoculation. Acidification of the culture broth preceeded extraction with ethyl acetate. The concentrated extracts were then treated with an excess of diazomethane, and pseudomonic acid (44) was purified as its methyl ester (58) by preparative TLC. Throughout the period of this work, the titre of recovered metabolite was found to be highly erratic.

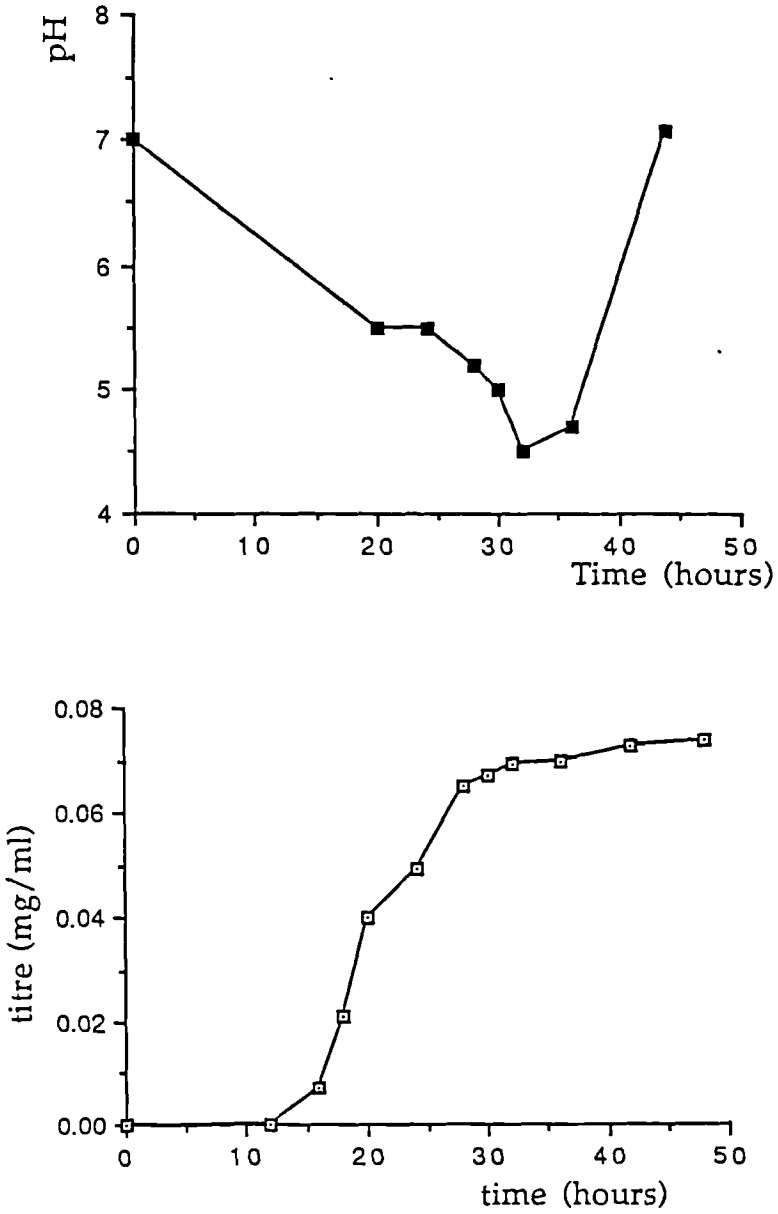


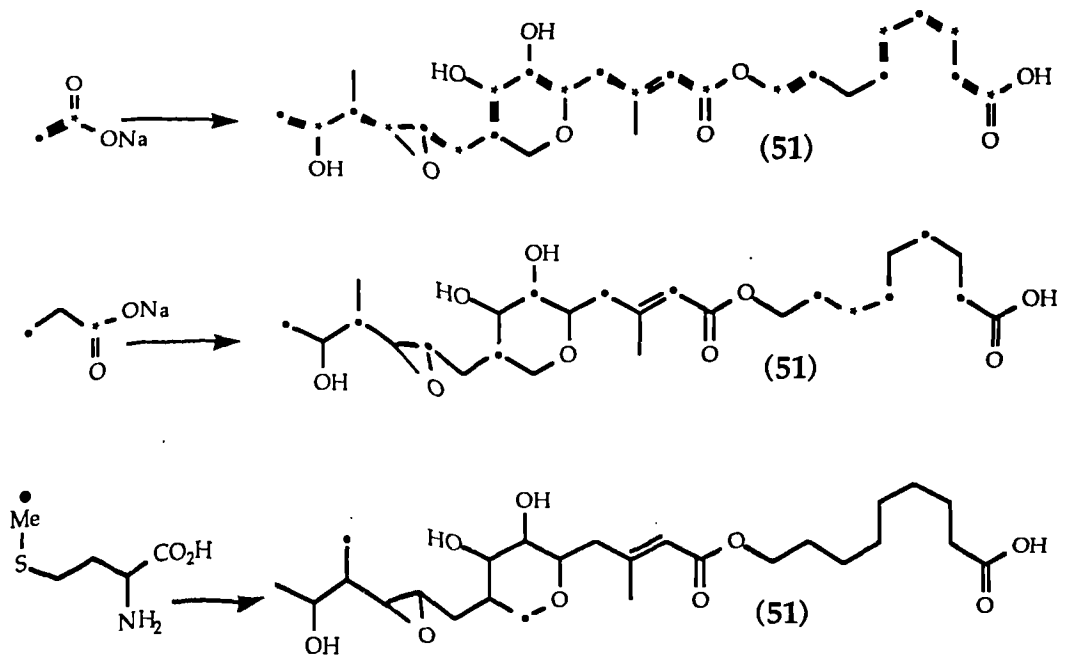
Figure 2.2.1: Growth production curve for pseudomonic acid (44) produced by *Ps. fluorescens* .

For biosynthetic studies it is necessary to have knowledge of the production phase of the metabolite (the idiophase).[23] Since pseudomonic acid (44) has a chromophore at 230nm, the culture broth was monitored for metabolite production by HPLC. The resultant growth-production curve is shown in figure 2.2.1, along with the pH variation of the medium during this time. This shows that metabolite production commences about 16 hours after inoculation of the secondary medium, with maximum production commencing soon after..

2.2.2: Previous studies on the biosynthetic origin of pseudomonic acid

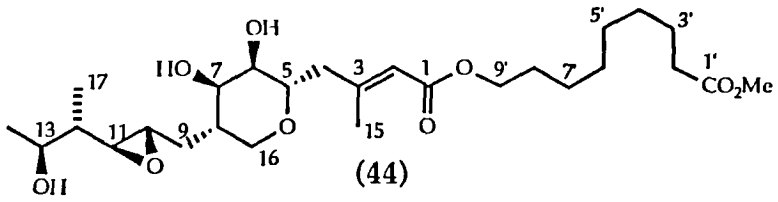
Despite the structural uniqueness of the pseudomonic acids, the oxidation pattern along the carbon back-bone appears to be consistent with a polyketide/fatty acid biogenesis.

To enable verification of this hypothesis by the utilisation of labelled compounds for nmr studies, the rigorous assignment of all resonances in the nmr spectra was required. The ^{1}H and ^{13}C nmr spectra of pseudomonic acid have been fully assigned,[24,25] and are shown in figures 2.2.2 and 2.2.3.



Scheme 2.2.1: Results of precursor feeding studies

δ	multiplicity	H
0.94	d	17
1.22	d	14
1.31-1.38	bs	4', 5', 6', 7', 12
1.44-1.98	bs	3', 8', 9
2.03	m	8
2.22	s	4ax, 15
2.31	t	2'
2.56-2.64	m	4eq
2.68-2.73	dm	11
2.81	m	10
3.46-3.59	m	16eq, 6
3.67	s	OMe
3.94-3.74	m	7, 16ax, 13, 5
4.07	t	9'
5.76	s	2



(44)

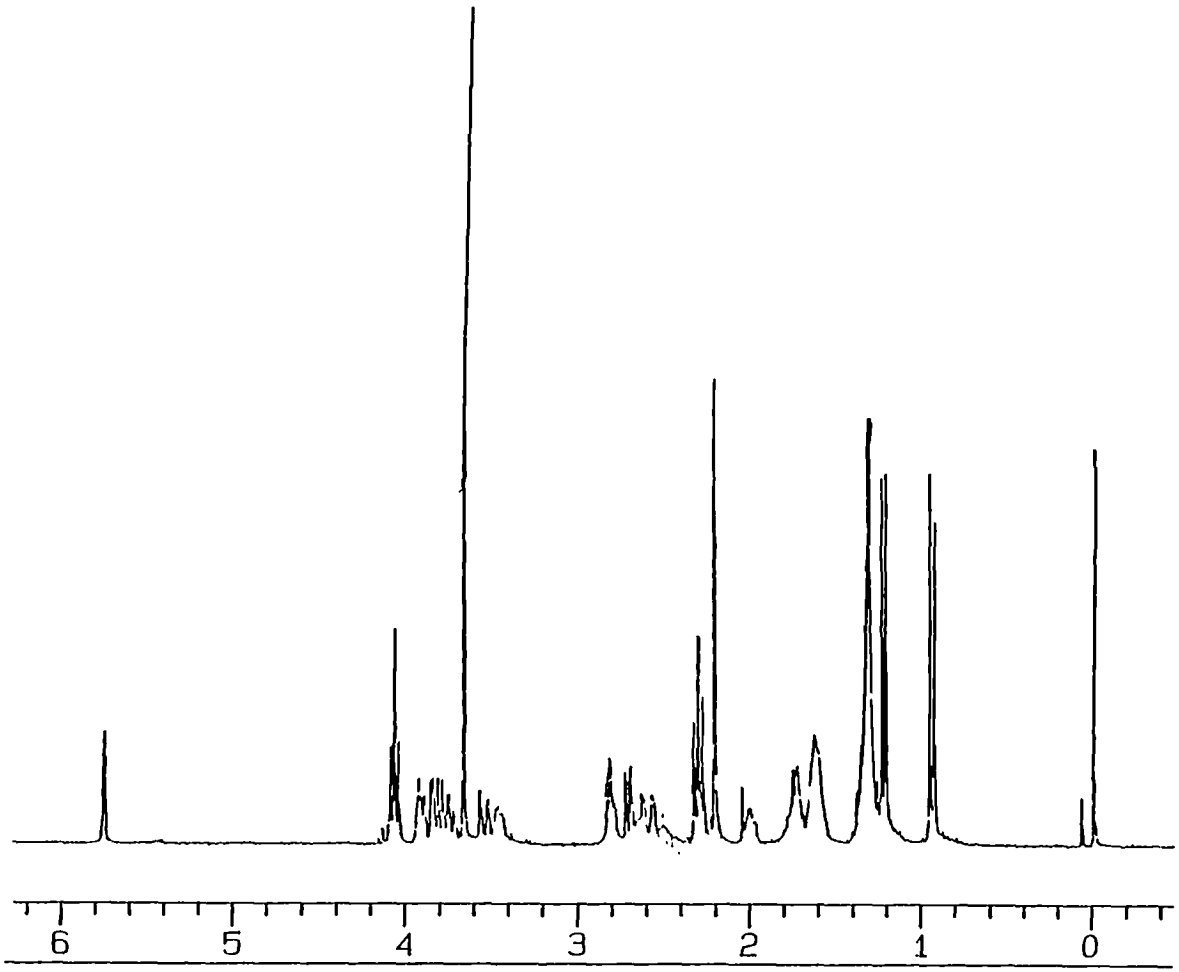
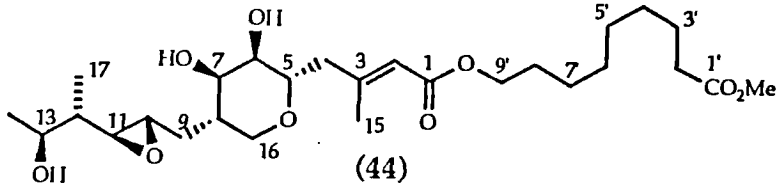


Figure 2.2.2: ^{1}H nmr spectrum of pseudomonic acid (44).

δ	C	δ	C
174.44	1'	51.53	OMe
166.77	1	42.86	12, 4
156.66	3	39.53	8
117.69	2	34.10	2'
74.88	5	31.56	9
71.46	13	29.11/29.05	4', 5', 6'
70.42	7	28.67	8'
69.02	6	25.97	7'
65.34	16	24.92	3'
63.85	9'	20.80	14
61.37	11	19.05	15
55.59	10	12.76	17



(44)

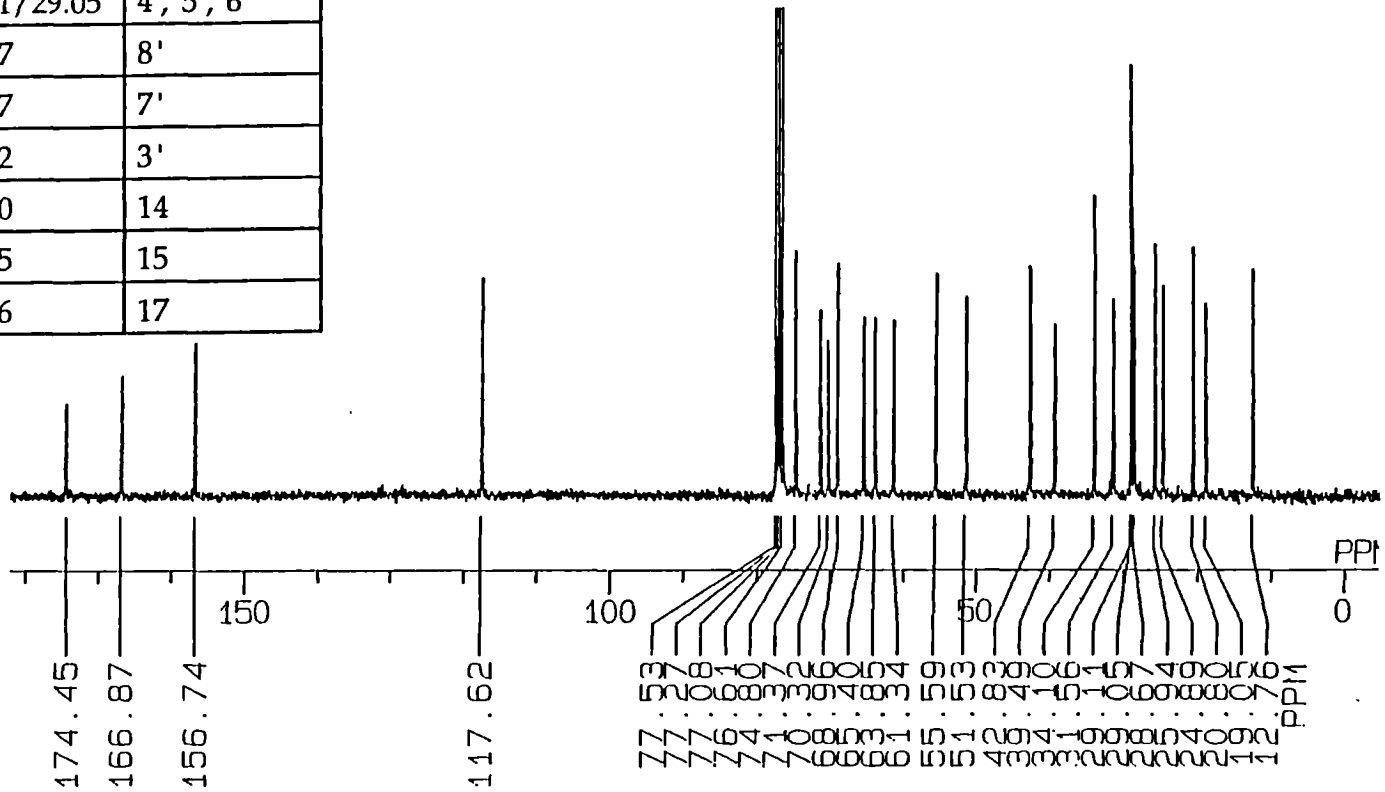
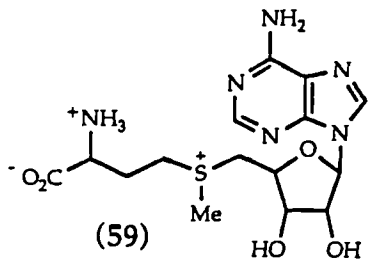


Figure 2.2.3: ^{13}C nmr spectrum of pseudomonic acid (44).

Feeding studies using variously labelled acetates propionates and methionine were carried out.[25] These results are shown in scheme 2.2.1. Radiolabelled mevalonate (**12**) was also fed, but no incorporation was observed. This was not surprising, as bacteria have not been shown to utilise this precursor. Radiolabelled formate was also tested as an alternative 'C_1-pool,' but the levels of incorporation were found to be very low.

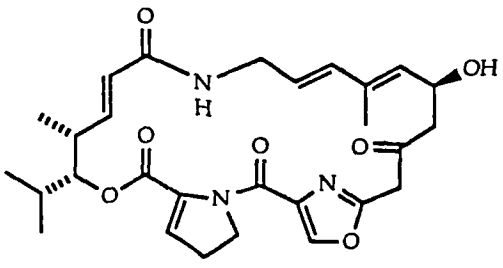
The incorporation of acetate units in a 'head to tail' manner along much of the carbon framework was found to be consistent with a fatty acid/poyketide biogenesis. There were, however, inconsistencies.

Whilst carbons 16 and 17 were found to be labelled from methionine, indicating branching methylation via S-adenosylmethionine (**59**), the branched C-15 methyl group was found to originate from C-2 of a cleaved acetate unit.

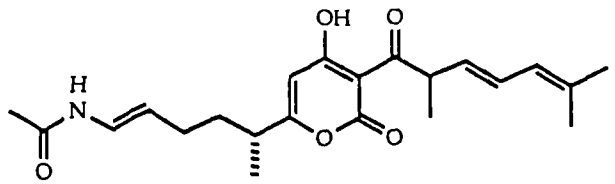


(59)

At the time, such a pathway was unique to pseudomonic acid (**44**) biosynthesis. Subsequent studies have shown a similar pathway to apparently be operating in the biogenesis of virginamycin M_1 (**60**),[26] myxopyronins (**61**),[27] aurantins (**62**),[28] and myxovirescin (**63**).[29]



(60)

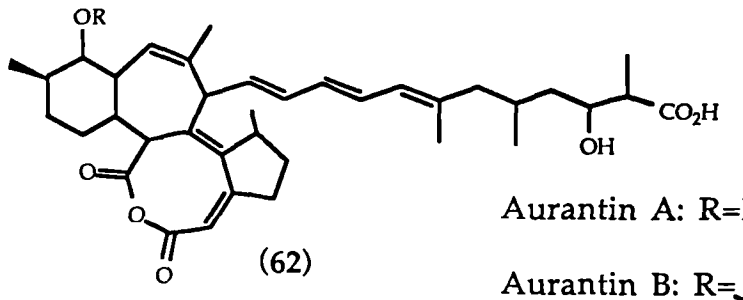


Myxopyronin A: R=Bu

Myxopyronin B: R=Pn

(61)

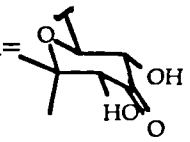
A further anomaly was discovered at C-7' in the aliphatic chain. This was found to be labelled by C-1 of a cleaved acetate unit, but the efficiency of incorporation appeared to be approximately one half that of other such derived carbons.

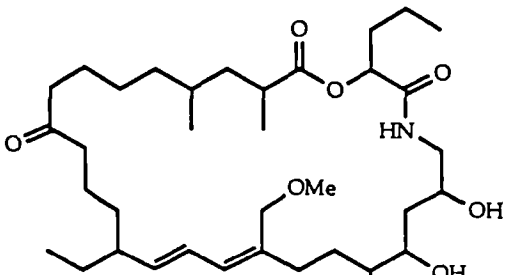


(62)

Aurantin A: R=H

Aurantin B: R=

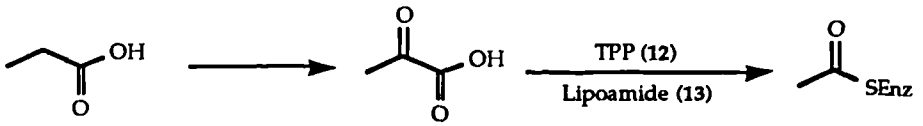




(63)

The origin of the aliphatic chain as a whole was also unusual. Intact acetate units appeared to be incorporated in a 'head to tail' manner at C-1'/C-2', C-3'/C-4', and C-5'/C-6' such that C-1' is derived from C-1 of acetate. C-8'/C-9' also appeared to be derived from an intact acetate unit, but the derivation of C-8' from C-2 of acetate indicated that the direction of acetate incorporation was the opposite to the rest of the aliphatic unit, with C-7' appearing to be the pivot for this reversal.

Studies using labelled propionate only served to exacerbate the confusion. [1-^{13}C]-propionate was found to specifically label the C-7' position. In contrast, the use of [3-^{13}C]-propionate gave rise to a metabolite showing a labelling pattern identical to that of [2-^{13}C]-acetate, indicating incorporation through acetate via α-oxidation to pyruvate (**11**), scheme 2.2.2 (see also scheme 1.1.6).



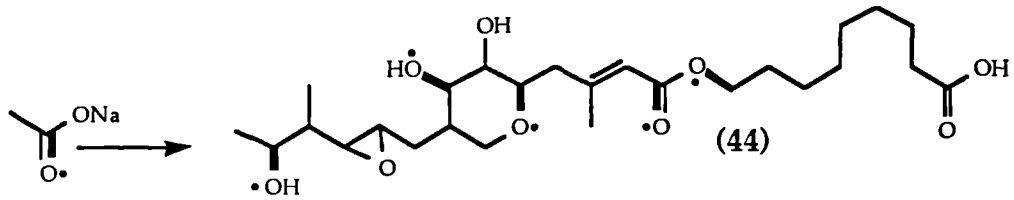
(11)

Scheme 2.2.2: Conversion of propionate to acetate via pyruvate **(11)**

From these early biosynthetic studies it was clear that although the metabolite (**44**) seemed to be of mainly polyketide origin, unusual pathways were being expressed.

2.2.3: Pseudomonic acid biosynthesis involving three sub-units.

The fact that both C-1 and C-9' were derived from the carbonyl carbon of acetate implied that the ester functionality was not the result of a Baeyer-Villiger type oxidation upon a pre-formed carbon chain. To confirm that the bonding oxygen of the ester moiety was acetate in origin, [1-^{13}C,^{18}O$_2$]-acetate was fed to *Ps. fluorescens*.[22] The results of this are shown in scheme 2.2.3, and confirm that the C-9′ to oxygen bond is indeed from an intact acetate unit.



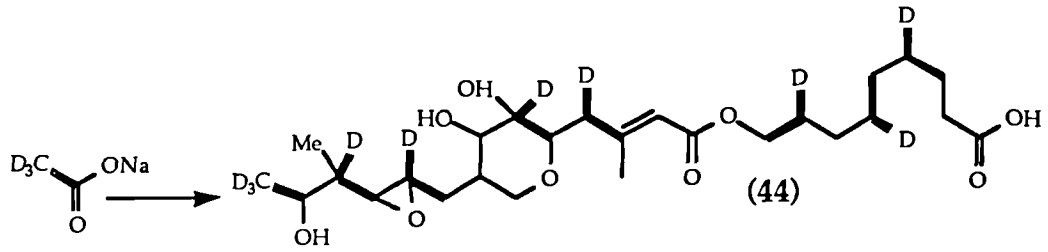
Scheme 2.2.3: Results of a feeding study with [1-^{13}C, ^{18}O$_2$]-acetate

That the oxygen at C-5 was retained from acetate implied that the C-5/ C-6 bond was not dehydrated and led to the postulation of a mechanism for the formation of the tetrahydropyran ring. This invoked a Michael type ring closure, as shown in scheme 2.2.4.[30]

Scheme 2.2.4: Mechanism of tetrahydopyran ring formation

To test this proposal, [1-^{13}C, 2-$^{2}H_3$]-acetate was fed to *Ps fluorescens*, the results[22,30] of which are shown in scheme 2.2.5. The lack of a β-shift in the ^{13}C nmr spectrum at C-7 indicated that deuterium from acetate was not retained at C-8. This was consistent with the proposed mechanism of ring formation.

The fact that the ester moiety appeared to be formed from two acetate units and not via a Baeyer-Villiger type mechanism indicated that at least two units were involved in pseudomonic acid (44) biogenesis. The labelling pattern previously described along the 9-hydroxynonanoic acid (45) moiety was inconsistent with a simple fatty acid biogenetic origin.

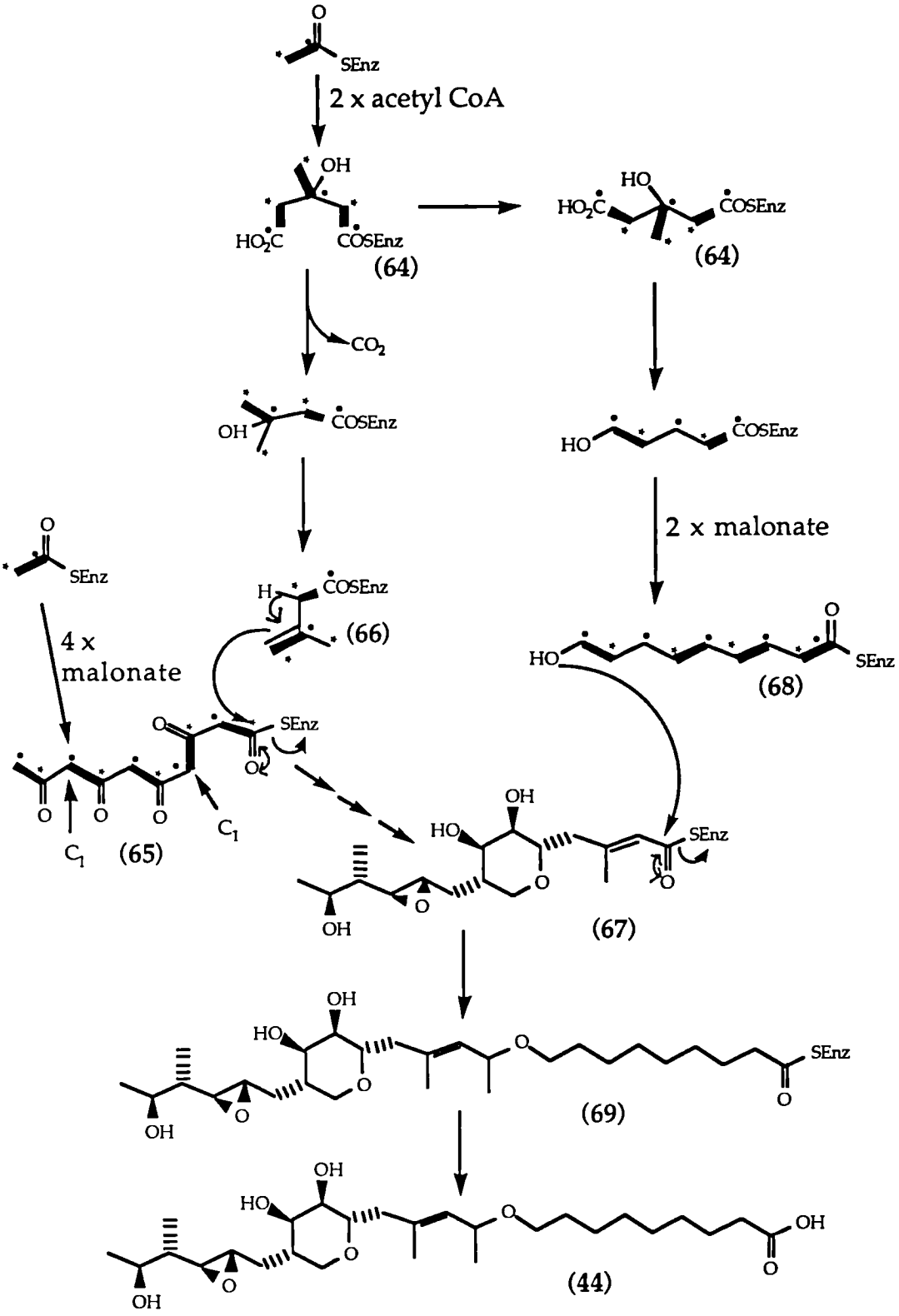


Scheme 2.2.5: Results of feeding study with [1-^{13}C, $^{2}H_3$]-acetate

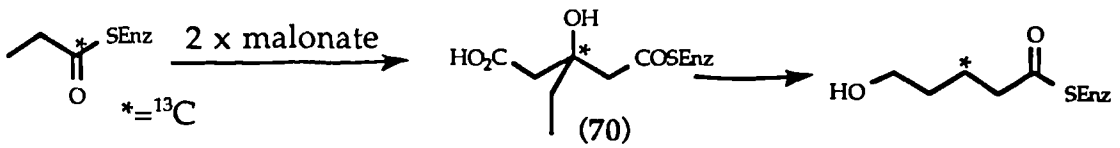
These anomalies were accounted for by proposing a triadic cascade biosynthetic pathway, invoking β-hydroxy-β-methylglutarate (64) as shown in scheme 2.2.6. Such a theory also gave a ready explanation for the origin of C-15 from the methyl carbon of acetate.

Thus, a polyketide derived C_{12} unit (65) was postulated to undergo allylic nucleophilic attack from a C_5 unit (66) derived from β-hydroxy-β-methylglutarate (64). The resultant α,β-unsaturated thioester (67) was postulated to undergo a further nucleophilic attack from a C_9 entity (68), again derived from β-hydroxy-β-methylglutarate (64) in conjunction with a fatty acid type chain elongation. Hydrolysis of the final thioester (69) gave release of pseudomonic acid (44).

To explain the labelling pattern at C-7', competition between β-hydroxy-β-methylglutarate (64) and β-hydroxy-β-ethylglutarate (70) was postulated, as shown in scheme 2.2.7. The incorporation of homomevalonate (71) (presumed to be derived from β-hydroxy-β-ethyl glutarate (70)) into juvenile hormone JH II (72) gave precedent for this suggestion.[31]

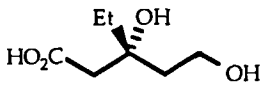


Scheme 2.2.6: Triadic cascade hypothesis

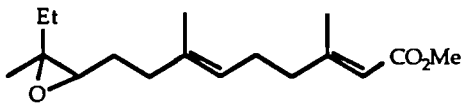


Scheme 2.2.7: β-hydroxy-β-ethylglutarate (**70**) as a precursor to the C_9 entity, originating from propionate.

These workers reported no data to verify the theory, however and it was not until some time later that the question was again addressed, when a report was made of the high incorporation of radiolabelled β–hydroxy–β–methylglutarate (**64**) into pseudomonic acid (**44**).[32] This was interpreted as good evidence for the above hypothesis, although no degradative studies were made to identify the position of incorporated radio-label



(**71**)



(**72**)

Further work using ^{13}C and $^{13}C_2$ labelled β-hydroxy-β-methylglutarate (**64**) for nmr studies confirmed that efficient label incorporation occurred. However, in both experiments the ^{13}C nmr spectra showed label incorporated throughout the molecule, with no evidence for any specific incorporation.[22,30] This implied that the administered compound had been degraded to acetate by catabolic enzymes prior to utilisation by the usual metabolic pathways. Further work confirmed this finding.[33]

In order to determine any involvement of malonate in the biosynthesis of pseudomonic acid (**44**), an initial experiment involving the adminstration of 250 mg of [2-^{13}C]-malonate to 250ml of inoculated medium was carried out. Incorporation into those sites already shown to be enriched by [2-^{13}C]-acetate was observed, with no evidence for any preferential incorporation at any site to indicate a starter effect. A second experiment involving 1.07g of [2-^{13}C]-malonate being fed to 125 ml of innoculated secondary medium was carried out. The results this time indicated some preferential incorporation of label into the 9-hydroxy nonanoic acid moiety, thus indicating a possible role for malonate in the biosynthesis of the aliphatic side chain.

2.3 : STUDIES ON THE ORIGIN OF 9-HYDROXYNONANOIC ACID.

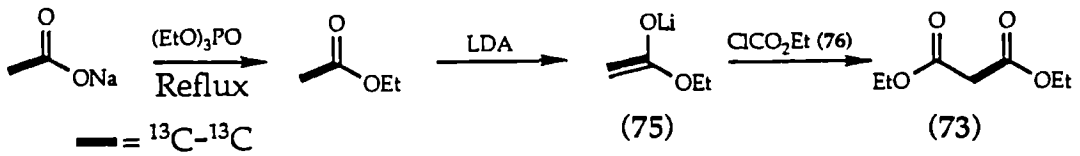
2.3.1 : The C-7' anomaly.

As has been previously discussed, the origin of C-7' is biosynthetically unique. Although studies utilising both terminal and carboxyl carbon labelled propionates were carried out, the use of [2-^{13}C] propionate was not investigated. To see if this could yield any further information, 250mg of sodium [2-^{13}C] propionate were administered to *Ps. fluorescens*. leading to the isolation of 17mg of metabolite (**44**).

The ^{13}C nmr spectrum obtained from this experiment is shown in figure 2.3.1. Enhanced signals are evident throughout the spectrum, corresponding to those carbons derived from the carboxyl of acetate. This is consistent with the [3-^{13}C]propionate result,[25] ie α-oxidation to pyruvate (**11**) prior to incorporation via acetate.

In an attempt to gain further information regarding the origins of the C-7'/C-8' bond, and thus C-7', [1,2-$^{13}C_2$] malonate (**73**) was synthesised. Owing to the inherent symmetrical nature of malonate, this is effectively a triply labelled moiety, with relative ^{13}C enrichments of 0.5:1:0.5. Should the bond under investigation originate from malonate, then associated ^{13}C-^{13}C couplings would be observed in the ^{13}C nmr spectrum.

For synthetic purposes, the label source chosen was sodium [1,2-$^{13}C_2$] acetate. As shown in scheme 2.3.1 this was converted to ethyl [1,2-$^{13}C_2$] acetate in 83% yield via reflux with triethyl phosphate (**74**) and distillation into a liquid nitrogen trap.[34] The lithium enolate (**75**) was formed and reacted with ethyl chloroformate (**76**) to produce diethyl [1,2-$^{13}C_2$] malonate (**73**) in 73% yield. Saponification of this for feeding studies[21] was achieved quantitatively with sodium hydroxide.



Scheme 2.3.1: Synthesis of diethyl [1,2-$^{13}C_2$]-malonate

Thus 250mg of the di-sodium salt was fed to *Ps. fluorescens*, leading to isolation of 13mg of methyl pseudomonate (**58**). The ^{13}C nmr spectrum is shown in figure 2.3.2. The resonance due to C-7' appears as a singlet, in

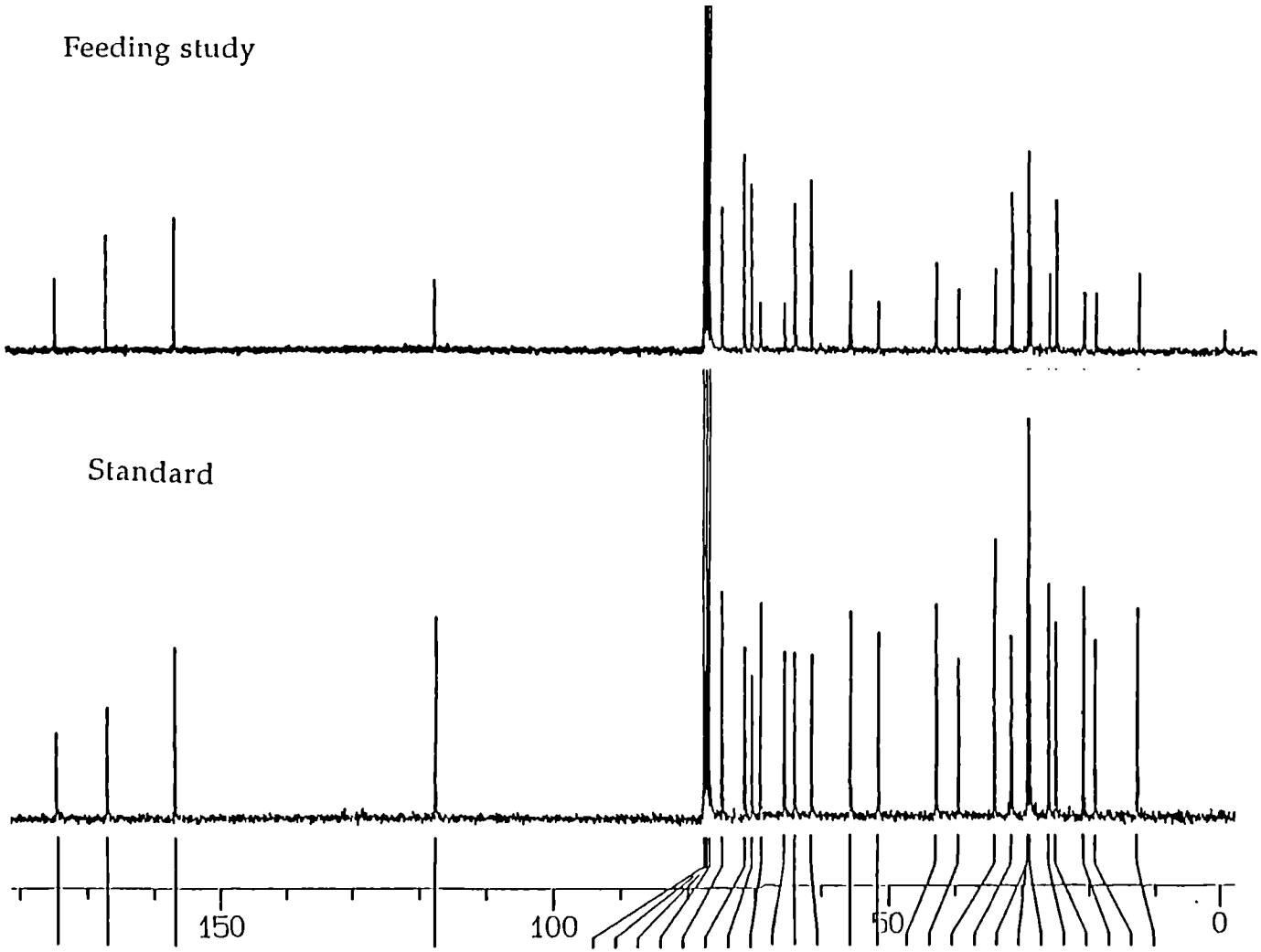


Figure 2.3.1: ^{13}C nmr spectrum of pseudomonic acid (**44**) after administration of [2-^{13}C]-propionate.

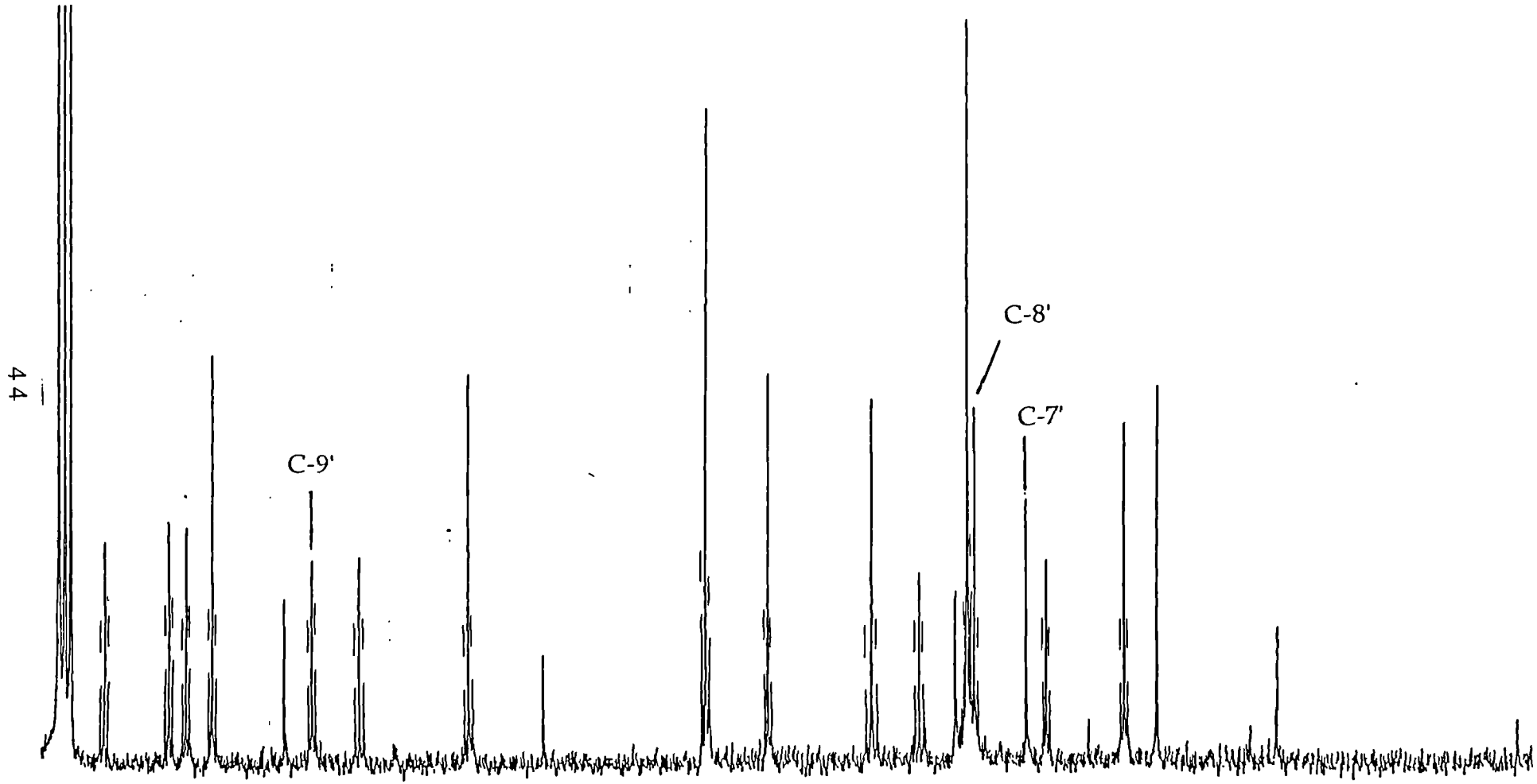
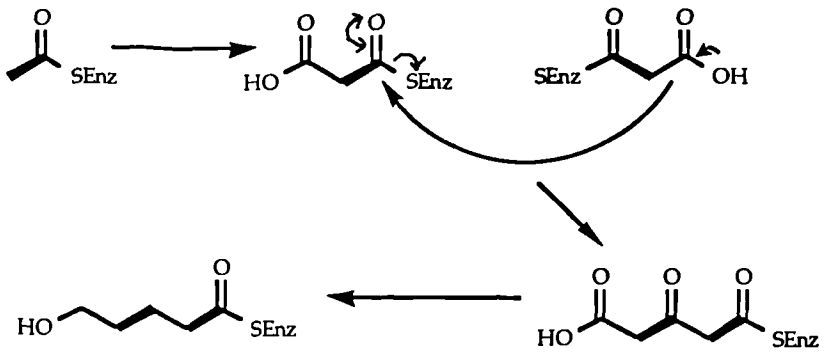


Figure 2.3.2: ^{13}C nmr spectrum of pseudomonic acid (44) after administration of [1,2-$^{13}C_2$]-malonate.

contrast to the C-8' signal, which is a triplet showing coupling uniquely to C-9'.

This demonstrates that the C-7'/C-8'/C-9' unit does not originate from an intact malonate starting group. Such a result was perhaps not unexpected, as for an intact malonate to be incorporated in this manner the bond labelled from [1,2-$^{13}C_2$] acetate would be expected to be C-7'/C-8', as shown in scheme 2.3.2.

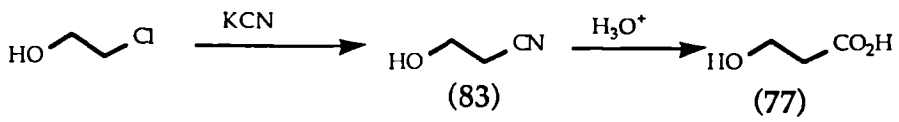


Scheme 2.3.2: Expected labelling pattern from acetate if malonate was the chain starter.

Further inspection of the aliphatic moiety led to speculation that 3-hydroxypropionic acid (77) may be a chain starting unit. Although not directly explaining the origin of C-7', such a possibility was further investigated.

2.3.2: 3-Hydroxypropionate as a chain starter.

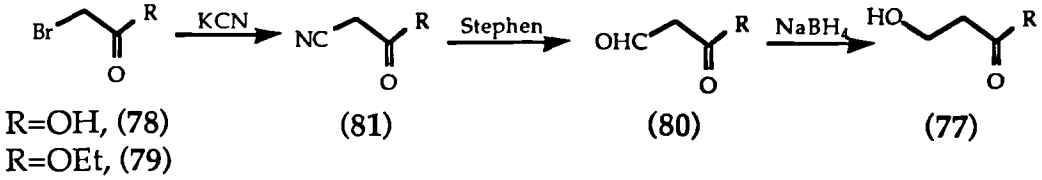
For these studies a synthesis of labelled 3-hydroxypropionic acid (77) was required. Potassium [^{13}C]-cyanide is a readily available source of label, and it was proposed to use this as shown in scheme 2.3.3.



Scheme 2.3.3: Proposed synthesis of 3-hydroxypropionic acid (77)

However, perusal of the literature showed that although the first step proceeded well under temperature controlled conditions,[35] the hydrolysis of the nitrile was low yielding and a large fraction (25%) of the product (77) was water.[36] Clearly this was undesirable.

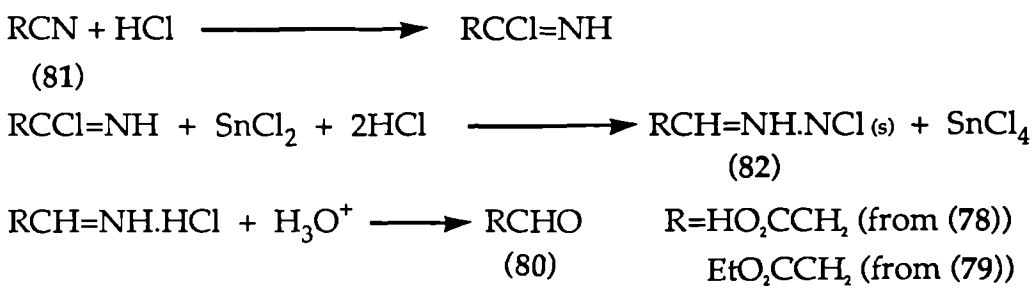
A more protracted route was briefly investigated, as outlined in scheme 2.3.4. Again the label source was to be potassium [^{13}C] cyanide, introduced via reaction with either bromoacetic acid (78) or its ethyl ester (79).[37] The possibility of subsequent specific reduction of the nitrile to the aldehyde (80) by the Stephen reaction,[38] and further reduction of this with sodium borohydride to yield the required alcohol was studied.



R=OH, (78) (81) (80) (77)
R=OEt, (79)

Scheme 2.3.4: Proposed synthesis of (77) via selective reduction

The Stephen reaction involves the addition of the nitrile to an anhydrous hydrogen chloride saturated ethereal solution of tin (II) chloride. An aldiminium hydrochloride salt (82) which precipitates out of the reaction mixture can be hydrolysed to the aldehyde (80) after isolation. The process is shown in scheme 2.3.5.



Scheme 2.3.5: The course of the Stephen reduction

The initial report on the reaction states the use of 1.5 moles of $SnCl_2$ per mole of nitrile, but under these conditions neither of the cyanoacetates (81) reacted as required. Increments in the amount of reductant were investigated,[39] however as much as 6 equivalents led to no discernible variance in the outcome of the reaction.

At this juncture re-investigation of the original nitrile hydrolysis was proposed, with the premise that for feeding studies the sodium salt would be required.[22] Thus 3-hydroxypropionitrile (83) was synthesised in 92% yield and subsequently hydrolysed with 1.05 equivalents of sodium hydroxide. However upon completion the mixture was not acidified but

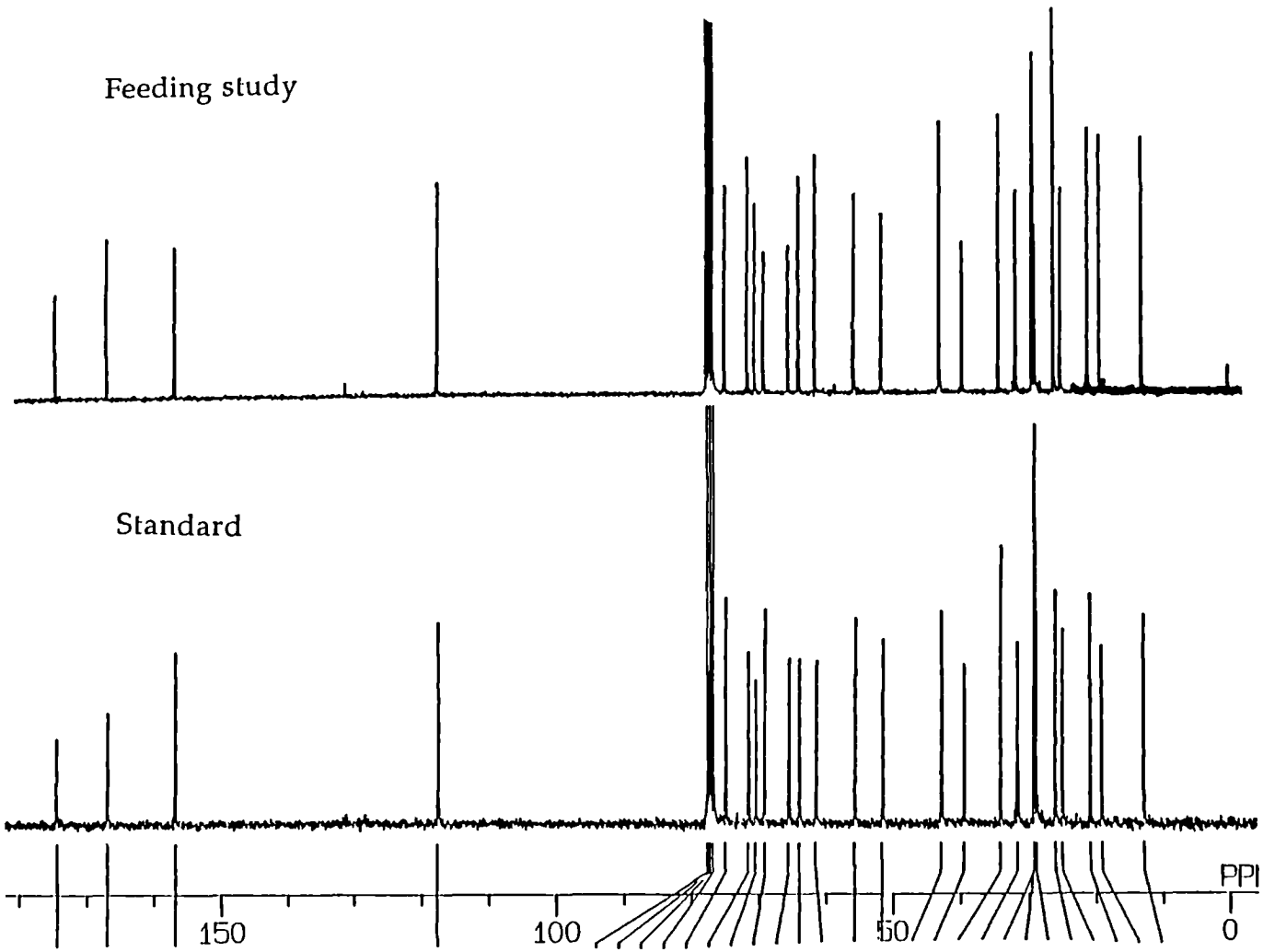
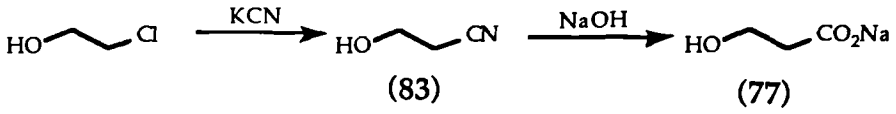


Figure 2.3.3: ^{13}C nmr spectrum of pseudomonic acid (44) after administration of [1-^{13}C]-3-hydroxypropionate.

concentrated *in vacuo* prior to lyophilisation. This yielded a quantitative yield of the required sodium 3-hydroxypropionate (77), scheme 2.3.6.

For feeding studies the sequence was repeated with potassium [1-^{13}C] cyanide thus producing the labelled form of sodium [1-^{13}C]-3-hydroxy propionate. Administration of 250mg of this to the bacteria produced, upon work-up, 29mg of methyl pseudomonate (58).



(83) (77)

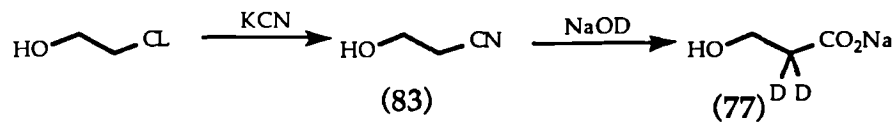
Scheme 2.3.6: Synthesis of sodium [1-^{13}C,2-$^{2}H_2$]-3-hydroxypropionate

Analysis of the ^{13}C nmr spectrum (figure 2.3.3) obtained from this showed enhancement of the resonance at δ 25.94, that due to C-7'. This, in turn, was the predicted site of incorporation of label from 3-hydroxypropionate (77), thus implying a possible role for this moiety in pseudomonic acid (44) biosynthesis.

As [1-^{13}C]-propionate gave comparable results,[25] however, it was necessary to determine whether C-1 of 3-hydroxypropionate (77) was incorporated via the same mechanism as that of propionate, or as an intact unit. For this, a bond labelled form of 3-hydroxypropionate (77) was required, with introduction of deuterium onto the carbon α to the labelled carbonyl being the chosen target.

Initially introduction of deuterium was attempted via proton-deuteron exchange in NaOD/D_2O. However neither the use of catalytic nor stoichiometric amounts of base led to significant exchange, even under refluxing conditions left overnight.

An alternative was sought via modification of the nitrile hydrolysis reaction. Thus the use of 1.05 equivalents of NaOD in D_2O led to hydrolysis with concomitant label introduction, scheme 2.3.7. Nmr analysis of the freeze-dried product showed complete exchange to have taken place, with the loss of signal at δ2.0 (C-2) and the triplet at δ3.55 (C-3) collapsing to a broadened singlet, figure 2.3.4.



(83) (77)

Scheme 2.3.7: Synthesis of sodium [1-^{13}C, $^{2}H_2$] 3-hydroxypropionate (77)

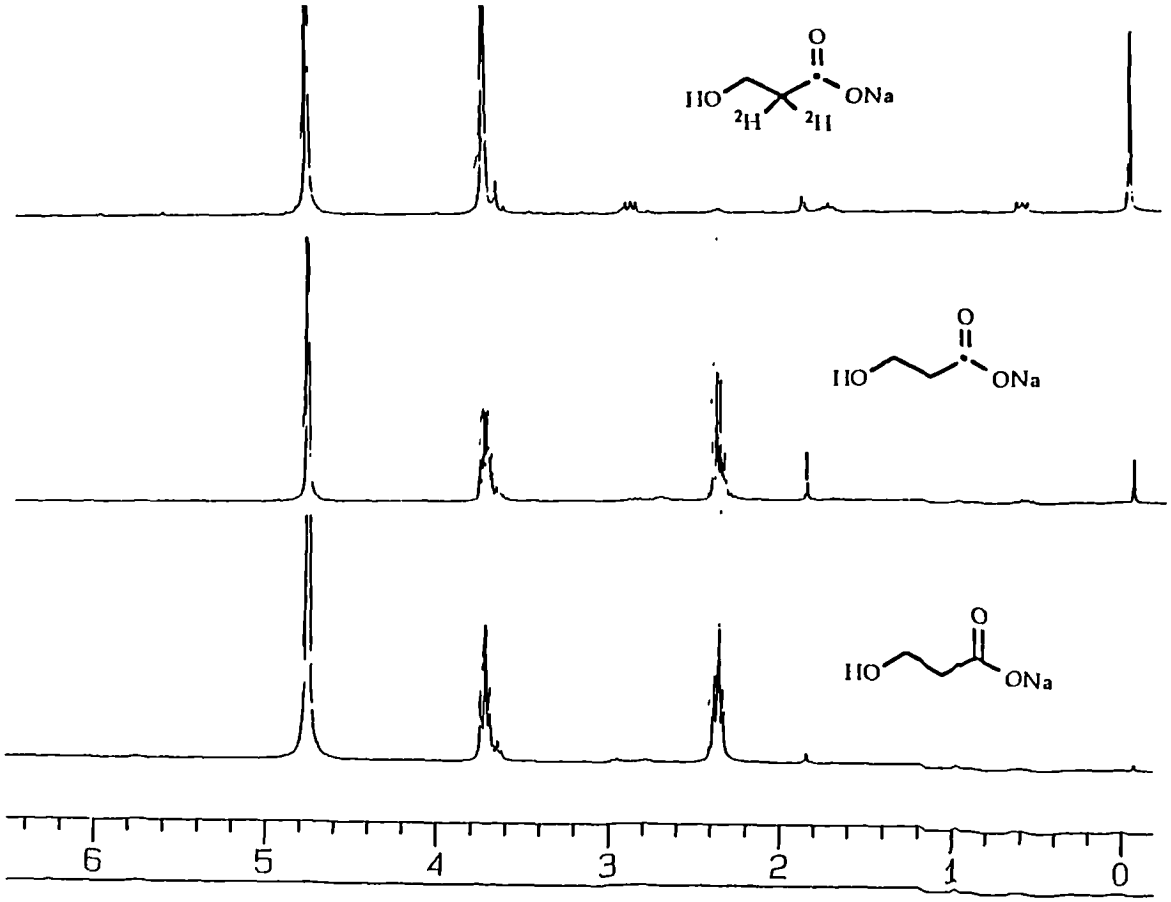


Figure 2.3.4: ^{1}H nmr spectra of labelled and unlabelled 3-hydroxypropionates.

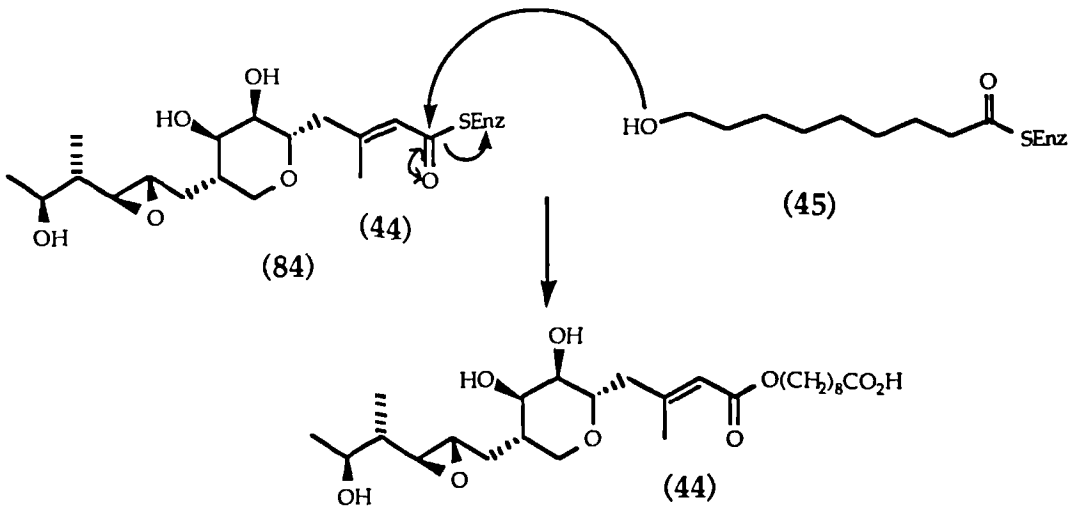
To *Ps. fluorescens* was administered 250mg of the doubly labelled material and after work up the 13mg of recovered methyl pseudomonate (58) was analysed by ^{13}C nmr spectroscopy, figure 2.3.5. No evidence for any incorporation of label was observed, either by enhancement of the C-7' resonance or by a ^{2}H (β) shift. Repetition of this experiment confirmed the result.

Although perhaps unlikely, a plausible explanation for this may be an isotope effect. If label incorporation was the result of decarboxylative cleavage and subsequent incorporation into a different (unknown) moiety prior to involvement in pseudomonic acid biosynthesis, then the ^{2}H label may have been deleterious to the action of enzymes upon the applied compound.

Duplication of the original singly labelled experiment was carried out in order to verify that result. However, ^{13}C nmr analysis of the resultant metabolite showed no evidence for label incorporation in contrast to the original experiment.

2.3.3: Pseudomonic acid biosynthesis involving two entities

Studies thus far have shown that neither a Baeyer-Villiger type oxidation, nor a triadic cascade biogenesis account for the labelling patterns observed in pseudomonic acid (44) biosynthesis. A simpler hypothesis invokes two distinct precursors to pseudomonic acid (44), monic acid (84) and 9-hydroxynonanoic acid (45), as shown in scheme 2.3.8



Scheme 2.3.8: Postulated biosynthesis of pseudomonic acid (44) from monic acid (84) and 9-hydroxynonanoic acid (45)

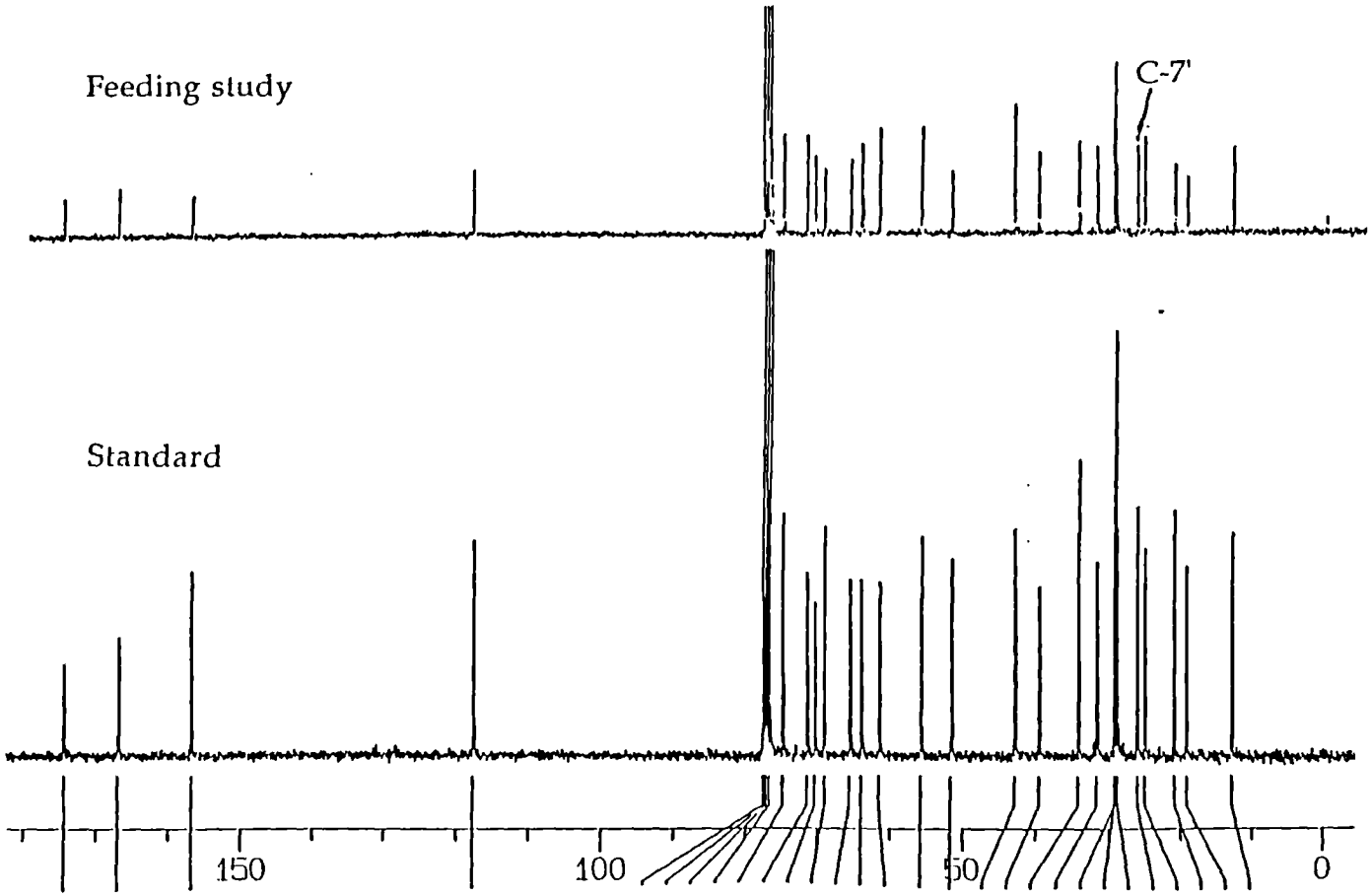


Figure 2.3.5: ^{13}C nmr spectrum of pseudomonic acid (44) after administration of [1-^{13}C, $^{2}H_2$]-3-hydroxypropionate.

In order to verify such a hypothesis, two basic approaches can be taken viz-à-viz administration of either labelled monic acid (84) or labelled 9-hydroxynonanoic acid (45) to the *Ps. fluorescens* in order to determine specific label incorporation.

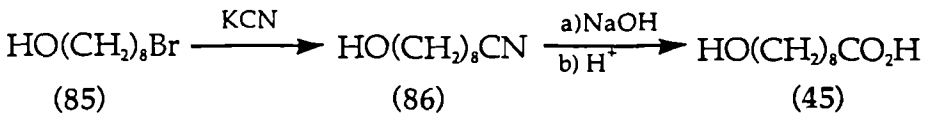
The first of these approaches was undertaken by Mantle and MacGeorge.[40] They obtained [16,17-$^{14}C_2$]-monic acid (84) via administration of radiolabelled methionine to bacterial cultures and subsequent saponification of the isolated labelled methyl pseudomonate (58). Upon feeding this to fresh cultures of the bacterium they found that essentially all the radioactivity remained in the medium, with the accumulated pseudomonic acid (44) being unlabelled.

Although this inability to traverse cell walls explains, at least partially, the low biological activity of monic acid (84),[13] it is uninformative regarding pseudomonic acid (44) biosynthesis. Indeed, it suggests that probing later stages on the biogenetic pathway of this metabolite may be fraught with similar difficulties.

The second of the above processes, namely the feeding of of labelled 9-hydroxynonanoic acid (45), is detailed below.

2.3.4: Synthesis of labelled 9-hydroxynonanoic acid (45)

As with the previously discussed synthesis of 3-hydroxypropionate (75), cyanide was chosen as the source for introduction of label. Thus carboxyl labelled 9-hydroxynonanoic acid (45) was synthesised by the route shown in scheme 2.3.9. Readily available 8-bromooctan-1-ol (85) was mixed with potassium cyanide under refluxing conditions overnight. Hydrolysis of the resultant 9-hydroxynonanitrile (86) produced the required product, in an overall yield of 78%.



(85) (86) (45)

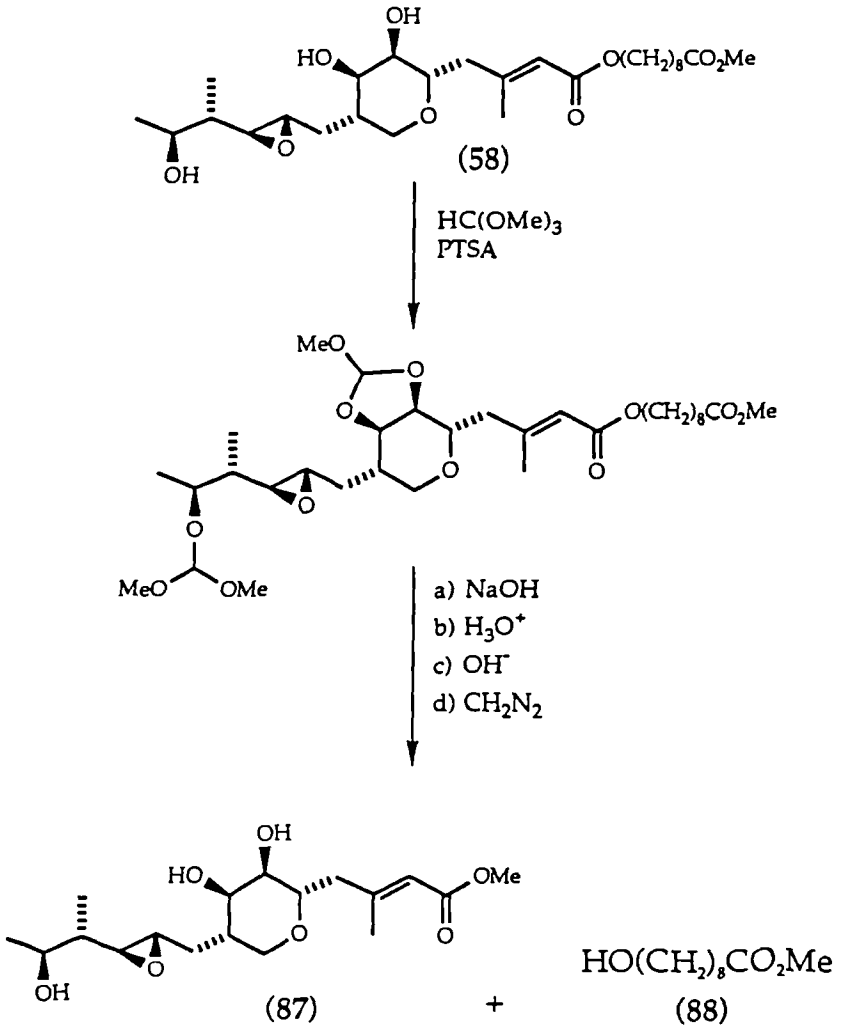
Scheme 2.3.9: Synthesis of 9-hydroxynonanoic acid (45)

2.3.5: Feeding studies using radiolabelled 9-hydroxynonanoic acid (45)

The use of radiolabels to study specific biosynthetic processes requires the precise position of any incorporation of label into a metabolite to be determined. This is usually achieved via degradative chemistry. The

hydrolysis of pseudomonic acid (44) to monic acid (84) has been described,[41] but this entails loss of the 9-hydroxynonanoic acid (45) by-product. Since, for these studies, this moiety would need to be recovered, a modification of the procedure was developed, as shown in scheme 2.3.10.

The methyl pseudomonate (58) isolated from the bacterial cultures was initially protected at the hydroxyl positions with trimethyl ortho formate, in order to prevent the rearrangements previously described (scheme 2.1.2). Saponification of this methyl ester, followed by hydrolysis of the monate ester, deprotection, treatment with diazomethane and then subsequent purification afforded the required methyl monate (87) and methyl 9-hydroxynonanoate (88). Unfortunately, this process was low yielding (36% for (87) and 17% for (88)).



Scheme 2.3.10: Degradation of methyl pseudomonate (58).

To cultures of the *Ps. fluorescens* were administered 10.41μCi of [1-^{14}C] 9-hydroxynonanoic acid (45). From these was isolated 34mg of methyl pseudomonate (58) exhibiting 82nCi of radioactivity, corresponding to an efficiency of label incorporation of 0.82%. Degradation of the metabolite was carried out as described to yield 3mg of methyl monate (87) and 1mg of methyl 9-hydroxynonanoate (88). Scintillation counting of these methyl esters showed both to contain significant amounts of radioactivity. The poor yields of the degradation process, and the low weights of the recovered products, meant that large errors were present such that accurate figures for the incorporation of activity into these moieties could not be calculated.

From these results it became obvious that some degradation of the administered 9-hydroxynonanoic acid (45) was taking place. In view of the inherent fatty acid nature of this moiety, this was not surprising. To probe the extent of this degradation it was decided to utilise stable labels and nmr analytical techniques.

2.3.6: Feeding studies using [1-^{13}C]-9-hydroxynonanic acid (45)

The required compound was synthesised as described using potassium [^{13}C]-cyanide as the label source, and 250mg were fed to *Ps. fluorescens*, which produced 29mg of methyl pseudomonate (58). Inspection of the ^{13}C nmr spectrum (figure 2.3.6) of this showed that although label had been efficiently incorporated into the metabolite, such incorporation was entirely non-specific via degradation to acetate through the action of β-oxidation enzymes.

Owing to the ubiquity of such enzymes, these competing biological processes are an inherent problem in studies of this nature. A number of inhibitors to fatty acid catabolic enzymes have been developed, of which one of the most effective is tetradecylthiopropionic acid (89).[42] It was hoped that concomitant administration of this inhibitor with labelled 9-hydroxypropionic acid (45) would reduce the *in vivo* degradation of this and therefore lead to information regarding the involvement of (45) in pseudomonic acid (44) biosynthesis.

$CH_3(CH_2)_{13}SCH_2CH_2CO_2H$

(89)

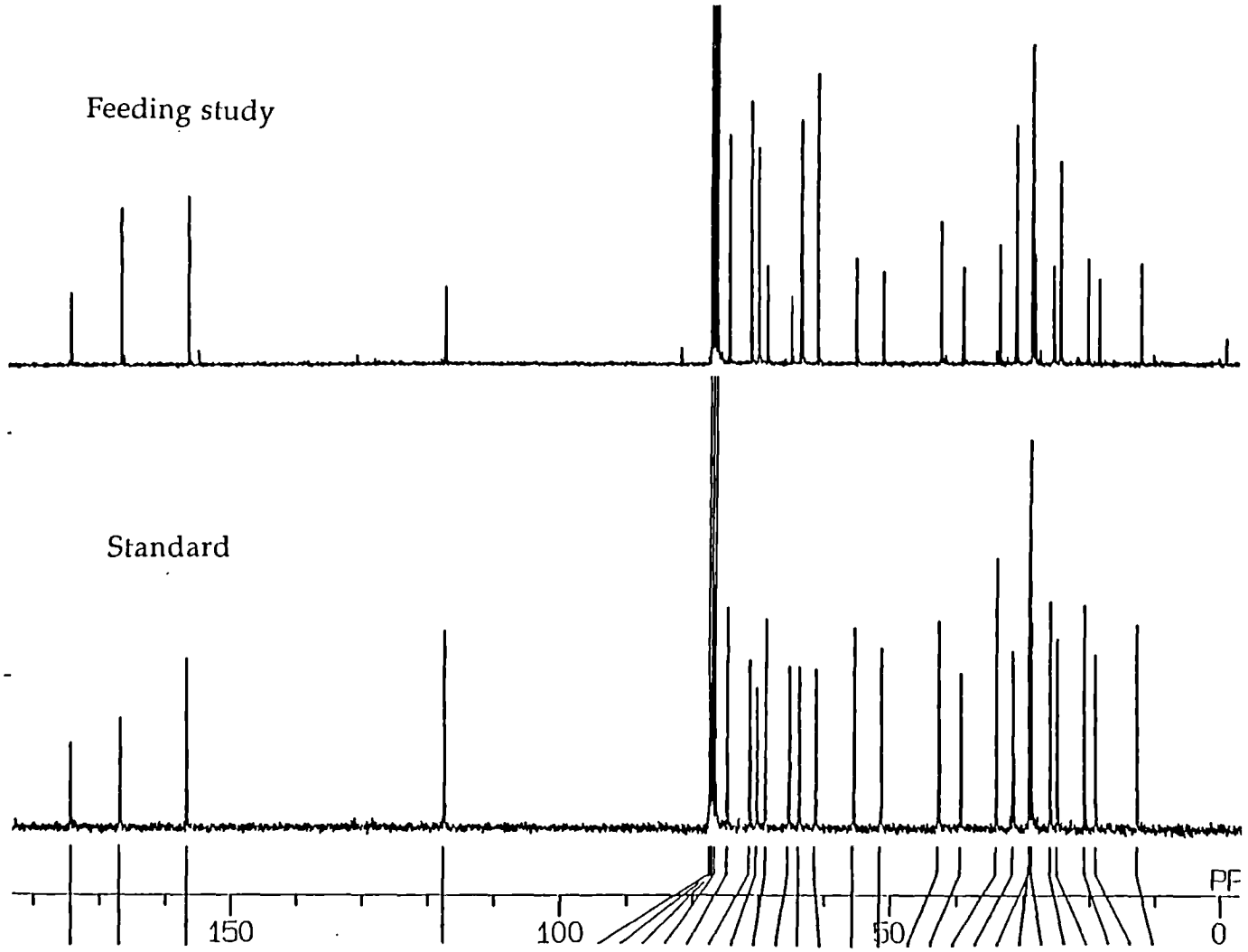


Figure 2.3.6: ^{13}C nmr spectrum of pseudomonic acid (**44**) after administration of [1-^{13}C]-9-hydroxynonanoic acid.

Prior to such an experiment it was necessary to ensure that the β-oxidase inhibitor did not also inhibit pseudomonic acid (44) biosynthetic processes. Thus 30mg of tetradecylthiopropionic acid (89) were added to 250ml of secondary media 20 hours after inoculation. Work-up as usual led to the isolation of 21mg of methyl pseudomonate (58). A control sample of *Ps. fluorescens* produced 23mg of metabolite, indicating that no inhibition had occurred.

For the main study, tetradecylthiopropionic acid (89) was fed in tandem with [1-^{13}C]-9-hydroxynonanoic acid (45). The usual work-up procedure led to the isolation of 17mg of methyl pseudomonate (58). Analysis of the ^{13}C nmr spectrum of this (figure 2.3.7) indicated that although incorporation of label had occurred, it was again via degradation of acetate with no evidence for any specificity.

Although these studies may be taken as evidence against the direct involvement of 9-hydroxynonanoic acid (45) in pseudomonic acid (44) biosynthesis, no firm conclusions can be drawn as a result of competitive catabolic processes. At this juncture it was clear that simple feeding studies were not going to yield definite information. It was therefore decided that the use of a 'cell-free' system should be investigated.

2.4: PSEUDOMONIC ACID BIOSYNTHESIS USING A CELL FREE SYSTEM

2.4.1: The use of cell free systems

The vast majority of intracellular chemical reactions are mediated by enzymes acting as highly efficient, commonly substrate specific, catalysts. As has been demonstrated, however, the *in vivo* study of one enzyme system can be thwarted by the presence of less selective enzymes.

Resolution of such problems may be possible by the isolation of enzyme activity for *in vitro* studies. Many examples of this approach exist in the literature, and two recent examples demonstrate the value of such experiments.

The biosynthesis of erythromycin (90) in *Streptomyces erythrea* (now re-named *Saccharopolyspora erythreus*) had been postulated to proceed via 6-deoxyerythronolide B (91) from the use of mutant strains.[43] Earlier work had shown that erythronolide B (92) was also an intermediate on the

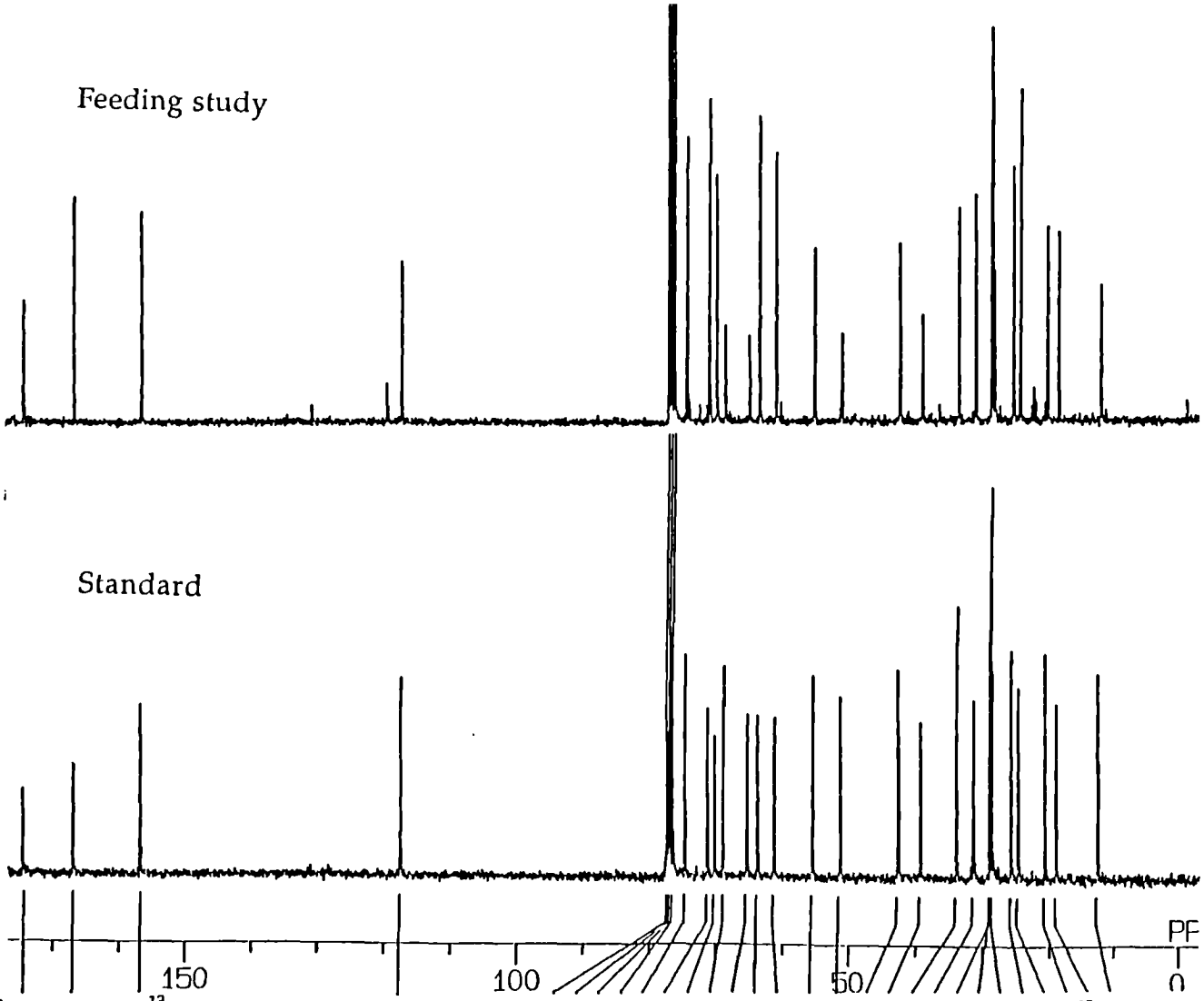
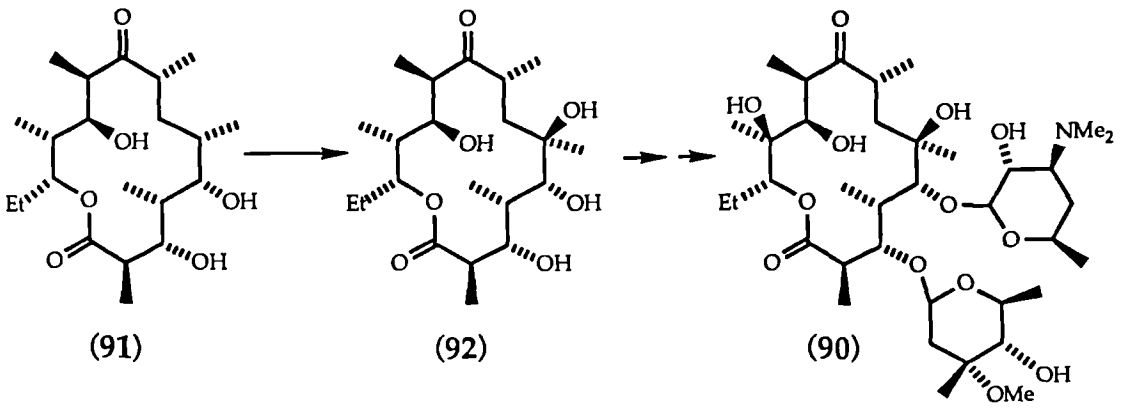


Figure 2.3.7: ^{13}C nmr spectrum of pseudomonic acid (44) after administration of [1-^{13}C]-9-hydroxynonanoic acid in tandem with a β-oxidase inhibitor.

biosynthetic pathway.[44] The hydroxyl group on C-6 of (92) was known not to be derived from acetate/propionate/ malonate precursors.

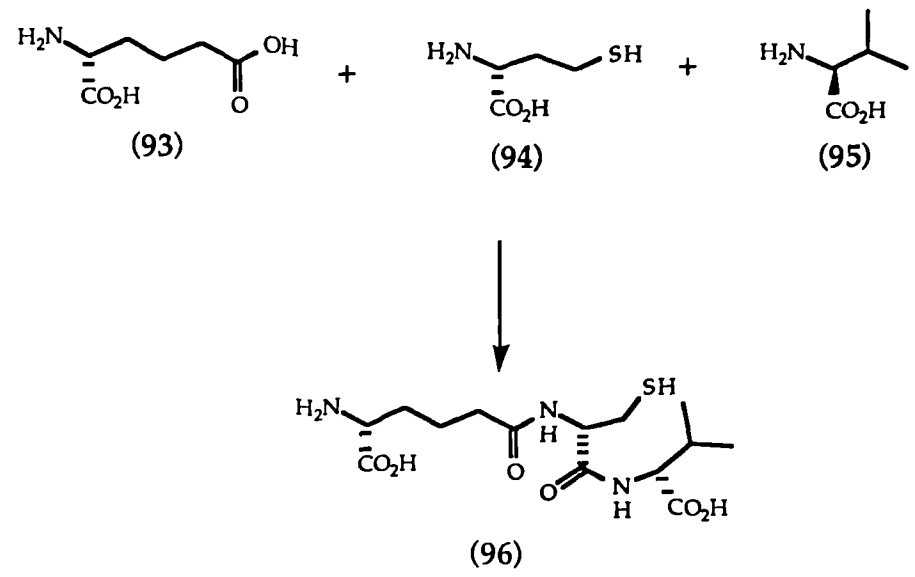


Scheme 2.4.1: Production of erythronlide B (92), a precursor to erythromycin B (90), from 6-deoxyerythronolide B (91) ,as shown in a cell-free system.

The demonstration that 6-deoxyerythronolide B (91) was the direct precursor to erythronolide B (92) was accomplished through use of a cell free system to which 6-deoxyerythronolide B (91) was administered leading to the production of erythronolide B (92),[45] as shown in scheme 2.4.1.

One of the most studied of all secondary metabolic processes has been that which leads to the penicillins and cephalosporins, the β-lactam antibiotics.[46] Although it was known that the biogenetic pathway began with the coupling of L-α-aminoadipic acid (93), L-cysteine (94) and L-valine (95) to form a tri-peptide starter unit, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (96), the process of the coupling itself was little studied until 1987. Cell free systems developed from *Cephalosporium acremonium*[47] and *Streptomyces clavuligerus*[48] showed that a single enzyme was responsible for the coupling process and that valine epimerisation occurs during formation of the tri-peptide, as shown in scheme 2.4.2.

It was hoped that verification of the hypothesis invoking two distinct precursors to pseudomonic acid (44) may be possible by the development of an appropriate cell-free system from the bacterium *Pseudomonas fluorescens*



(93) (94) (95) (96)

Scheme 2.4.2: The formation of the tri-peptide starter unit for the β-lactam biosynthetic pathways.

2.4.2: Lysis of cell membranes.

To produce a cell free system at least partial destruction of the cell walls to enable release of enzyme is required. A variety of methods have been developed for this.[49]

a) Enzymatic

The use of enzymes to digest cell walls is usually achieved by the use of lysozyme, which is extracted from egg-whites. This enzyme hydrolyses the β-1,4-glycosidic bonds of the mucopeptide of bacterial cell walls. In general, Gram positive bacteria are most susceptible to such treatment.

Other enzymes less frequently used are microbial gluconases and lysostaphin.

b) Chemical

The short exposure (20 minutes) of bacteria to alkaline conditions (pH 12) has been used for cell wall lysis, but the success of such an approach requires that any released protein be alkali stable.

Detergents, both ionic or non-ionic, have also been used. Ionic detergents are the most reactive, but are more prone to give rise to enzyme denaturation as a result. The presence of detergent also leads to an increased number of steps should enzyme purification be desired.

c) Physical

The most widely employed technique, especially for small scale work, is that of sonication. A cellular suspension of cells is subjected to repeated bursts of high energy sound waves, which leads to the piercing and ultimately the destruction of the cell walls

Another oft-used method is osmotic shock. This involves washing the cells free of growth medium prior to suspension of the cells in a buffered 20% sucrose medium. After equilibration the cells are harvested and rapidly resuspended in water at 4°C. This yields 4-8% of the total bacterial protein.

Grinding with abrasives, for example glass beads or sand, is a frequently used method, leading to total destruction of the cell walls.

A different technique is shearing, either in solid or liquid phase. This involves the extrusion of a sample through a narrow orifice after subjection to high pressures. For liquid suspensions on a small-scale, a continuous French press is often used.

With the exception of osmotic shock methodology, physical lysis techniques cause the generation of large amounts of heat which must be efficiently and rapidly dissipated so as to avoid enzyme denaturation.

2.4.3: Determination of cell lysis.

For cell free studies extensive rupture of the cellular membrane needs to have occurred to allow efficient release of protein. A simple qualitative test for this is a reduction in the viscosity of the cell suspension after treatment.[50] A more rigorous test is possible through microscopic observation of changes in the cell structure. Either visible microscopy, for the study of cell colonies, or electron microscopy, for the study of individual cells, may be used. Measurement of the amount of protein release can also be used to quantify any cell membrane disruption.

2.4.4: Lysis of *Pseudomonas fluorescens*.

For these studies a high producing strain of *Ps. fluorescens* (PF3/N/2), that had been developed from the usual strain,[51] was used with the assumption that higher titres of enzyme would be present. This was grown under the same conditions used for the normal producing strain until metabolite production was shown to have commenced by HPLC

assay of the medium. The cells were then harvested by centrifugation, and washed prior to re-suspension in buffer for cell fissure experiments.

The nature of the enzyme system under investigation was unknown and so it was decided that mild lysis techniques would be investigated to reduce the possibility of protein denaturation.

The method initially investigated was sonication. The cells were suspended in a buffer of pH 7.6 prior to treatment. Sonication led to a suspension of slightly reduced viscosity. Sonication on cellular suspensions buffered to pH 7.2 and 6.9 was also carried out.

Lysozyme was also utilised for cell lysis. Initially the direct addition of the enzyme to a buffered suspension at pH 7.6 was tested. However as *Ps.fluorescens* is a Gram negative bacterium it was unlikely that this would lead to useful cell rupture. In order to aid the enzyme the cellular suspension was pre-treated with EDTA.[52] It was hoped that such a chelating agent would remove metal cations from the outer membrane leading to a weakening of the cell wall thus rendering it more susceptible to enzymatic lysis.

Finally, liquid shear by passage though a French press was also investigated.

Optical microscopy was used to analyse the effect of such treatments on the cells. In all cases it was observed that with respect to an untreated sample, cellular coagulation occurred. Staining of dried sample slides gave no further information. Thus, although it appeared that a change had occurred it was not possible to quantify the nature or extent of this. The samples were submitted for electron microscopy, but the time needed for such analysis meant that studies proceeded without definite confirmation of cell rupture. The results of the electron microscopy studies are discussed in appendix 2A.

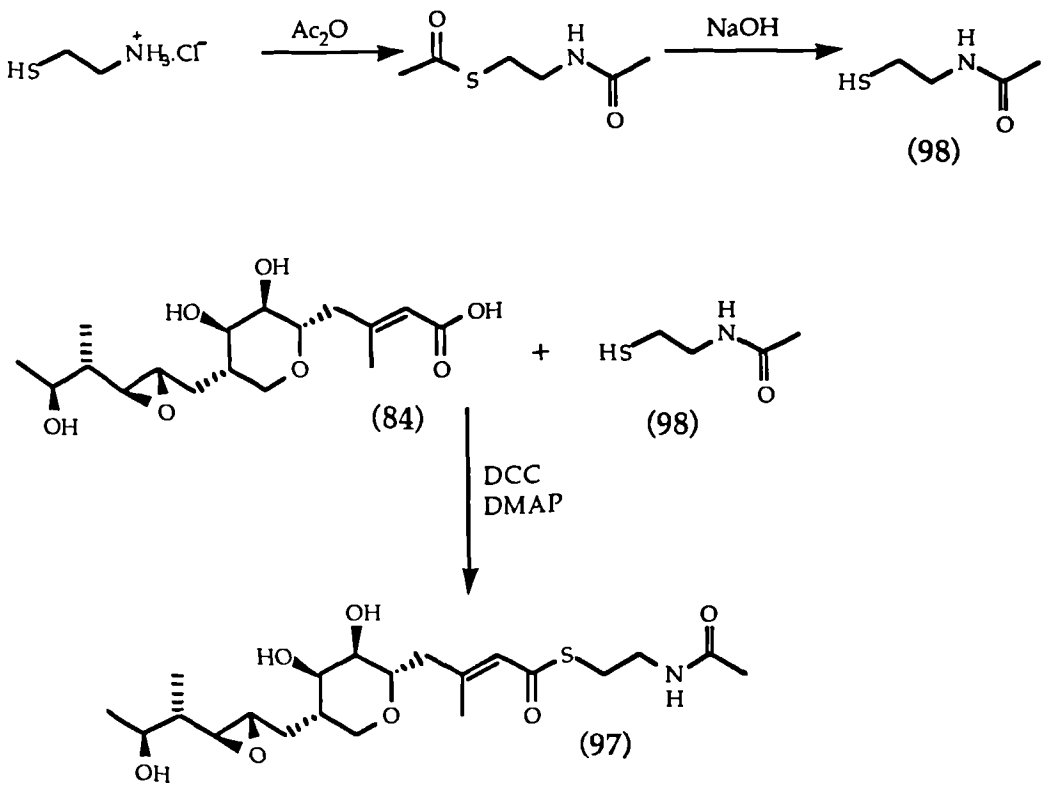
2.4.5: Cell free studies

In order to test the working hypothesis two reactions were chosen for investigation. The first of these was that between monic acid (84) and 9-hydroxynonanoic acid (45) based on the premise that this was the formal reaction being postulated within the hypothesis.

In biological systems, however, reactants are frequently utilised in an activated form, a common example of this being the activation of acetate as acetyl CoA (9). Such thioesters are frequently found as a source of

activation in biological systems. The studies into the stereospecificity of fatty acid biosynthetic processes discussed in chapter 1 showed that N-acetylcysteamine thioesters (33) acted as mimics for this type of activation. The second reaction chosen for observation, therefore, was that between the N-acetylcysteamine thioester of monic acid (97) and 9-hydroxynonanoic acid (45).

This was synthesised by the use of DCC/DMAP coupling,[53] N-acetyl-cysteamine (98) being easily synthesised from readily available materials,[54] as shown in scheme 2.4.3. The solubility of monic acid (84) in organic solvents was found to be poor, with the exception of simple alcohols (the use of which would lead to oxy-esters as the main product). Solvation of monic acid (84) in tetrahydrofuran led to a turbid solution and so this was employed as the reaction medium. However under these conditions the yield of the required thioester (97) was only 18%. As monic acid (84) was readily available, the poor yield was deemed to be sufficient for these studies.



Scheme 2.4.3: Synthesis of N-acetylcysteamine (98) and the N-acetyl cysteamine thioester of monic acid (97).

For each set of experiments, fresh cell preparations were used for lysis and the resultant suspensions used immediately after treatment. Further experiments were carried out using the supernatant after centrifugation of lysed samples in order to determine the extent of any membrane association requirement.

The initial ratio of monic acid (84) or thioester (97) to 9-hydroxy nonanoic acid (45) was 1:3, with the acid/thioester at 5mmol concentration. In later experiments the ratio was increased to 1:5 with an acid/thioester concentration of 2mmol. Reaction times ranging from 2 to 20 hours were tested, under static and mixing conditions.

Under these conditions, and using all the possible permutations of available cell free 'enzyme,' no production of pseudomonic acid was observed by HPLC assay.

Within the last decade, Klibanov has pioneered the use of 'anhydrous' organic solvents as media for the use of enzymes.[55] Following the lack of success of the previous experiments it was decided that the use of freeze-dried enzyme preparations should be investigated to see if this would yield any information. One advantage of such an approach was the use of a further reaction, namely the hydrolysis of pseudomonic acid (44) to monic acid (84) and 9-hydroxynonanoic acid (45). This was postulated on the basis that esterases are known to catalyse both the formation and hydrolysis of esters.[55]

For such studies three anhydrous solvents were chosen; 1,4-dioxan, tetrahydrofuran and acetonitrile. A small amount of water is often required for such reactions, to ensure retention of enzymic structure. Thus experiments involving the presence of 0.5%, 0.1% and 0% water were tested. One of the problems with this approach was the parity of substrate to solvent. Where one of the substrates was not highly soluble in the solvent of choice, it was added in another of the anhydrous solvents. In all cases, rapid shaking was used to ensure thorough mixing. Again, reaction times varied from 2 to 20 hours for all possible permutations.

No evidence for pseudomonic acid (44) production or degradation (ie monic acid (84) production) in any of the experiments was observed by HPLC assay.

There exists a number of plausible explanations for these results. One possibility is simply due to poor release of protein. Another reason may arise from denaturation of enzyme owing to either inherent extra-cellular instability or oxidation under atmospheric conditions. Experiments under

reducing conditions were not carried out. The enzyme may require a number of co-factors for efficient catalytic activity which would have been unavailable under the conditions investigated.

Further explanations may lie in the actual timing of the esterification (and thus the species involved) with respect to other steps on the biosynthetic pathway to pseudomonic acid (**44**).

2.5: SEQUENTIAL PSEUDOMONIC ACID (44) BIOSYNTHESIS.

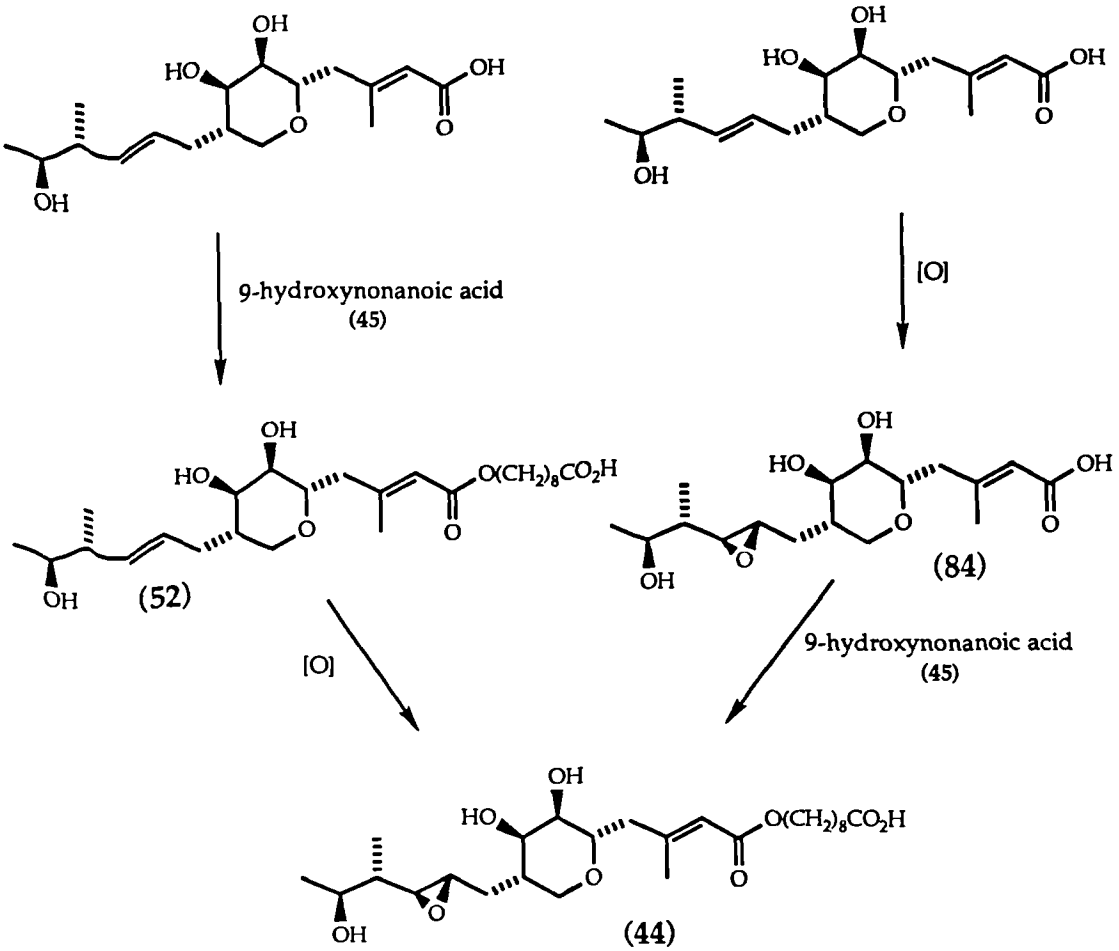
2.5.1: Precursors to monic acid (45)

The esterification hypothesis postulated the reaction partners to be monic acid (**84**) and 9-hydroxynonanoic acid (**45**). However, it is possible that precursors to either of these species are the actual substrates, with further modifications subsequent to this giving rise to the final metabolite. The timing of the ring closure step with respect to any esterification process is also unknown.

A co-metabolite of pseudomonic acid (**44**) is pseudomonic acid C (**52**). This has an identical carbon back-bone to (**44**) but lacks the epoxide functionality, possessing a *trans*-double bond instead. It is more likely that epoxidation of the double bond leads to pseudomonic acid (**44**) as opposed the de-oxygenation of pseudomonic acid (**44**) leading to pseudomonic acid C (**52**). However such an epoxidation step may occur prior or subsequent to any esterification, as shown in scheme 2.5.1. The existence of pseudomonic acid C (**52**) does not discriminate against either possibility.

The events leading to the origin of C-15 from C-2 of acetate[25] are to be discussed in chapter three. However, such a process could occur in a relevant precursor to either monic acid (**84**) or pseudomonic acid (**44**). The timing of this step with respect to ester formation is again unknown.

In order to ascertain information regarding the sequence of steps on the pathway to pseudomonic acid (**44**), some preliminary experiments have been under taken.



Scheme 2.5.1: Relative timing of epoxidation and esterification steps on the biogenetic pathway to pseudomonic acid (**44**).

2.5.2: The use of oxidase inhibitors and mutant strains

The ordering of biosynthetic events can determined by two methods, namely the use of blocked mutant strains of the producing organism or the use of enzyme inhibitors. Potentially the most profitable is the use of blocked mutants. This involves genetically altering the producing organism to produce new strains of non-producing organism. If a large number of altered organisms are screened, then it is possible that some of these new organisms will produce new metabolites that are intermediates on the biosynthetic pathway to the natural product under consideration. In these cases the mutation will have blocked the subsequent step on the biosynthetic pathway. From structural comparisons it may be possible to predict the order of events leading to the final metabolite, but this may not always be obvious.

However if a number of mutants have been produced then co-synthesis experiments can be used to determine the sequence. If one non-

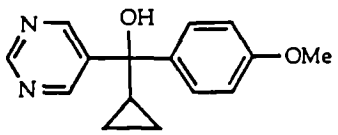
producing mutant accepts the intermediate excreted by another mutant such that the first mutant now produces the original metabolite, then it can be deduced that the pathway in the acceptor mutant has been blocked earlier than in the second (excreting) mutant. Investigation of all the possible permutations from such mutants leads to a precise knowledge of the ordering of events along a biosynthetic pathway.

Studies into the genetics of pseudomonic acid (44) biosynthesis in *Ps. fluorescens* have produced a number of non-producing mutant strains of the bacterium.[57] As part of this work it was of interest to determine whether any of these expressed any new metabolites that may be of biosynthetic interest. The mutant strains were cultured under the usual conditions and then assayed by HPLC for pseudomonic acid (44), monic acid (84) and then any new peaks under a variety of solvent systems. However, no peaks were seen.

The genetic work is continuing and more mutant strains are being produced. Further assay of these may give useful results. A further experiment worth investigating as part of such studies is the 'spiking' of the culture broth with a trace of radiolabelled methionine. Any uptake of this will be detectable and, in conjunction with preparative HPLC techniques, new metabolites may be discovered.

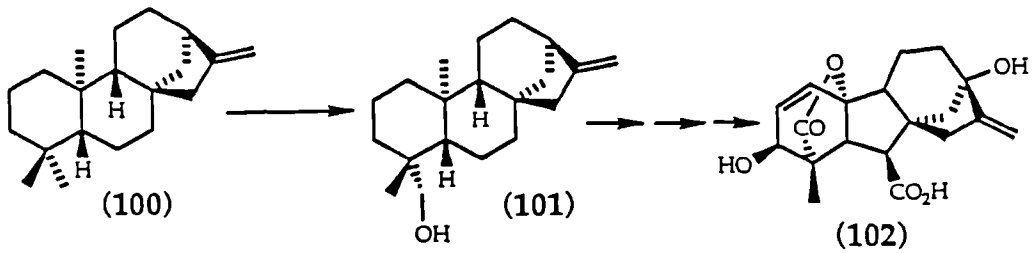
The second method used for gaining information regarding the sequence of events on a biogenetic pathway is by the use of enzyme inhibitors. In many cases these are only useful for probing late biosynthetic events, as early inhibition is unlikely to yield interesting structures of biosynthetic use.

Pseudomonic acid (44) has two oxygen atoms known not to be derived from acetate,[22,30] the epoxide oxygen and the hydroxyl on C-6. These are assumed to be of atmospheric origin, although experiments designed to confirm this did not produce enough metabolite for the relevant analysis to be performed. If such an assumption is correct, the oxidase involved is likely to be a cytochrome-P_{450} type enzyme.[58] A known inhibitor of this species of enzyme is ancymidol (99).[59] In possessing both lipophilic and heteroatomic moieties, this can reversibly bind to the lipophilic domain and heam-iron of cP_{450} enzymes, thus displacing both the substrate and molecular oxygen.[60]



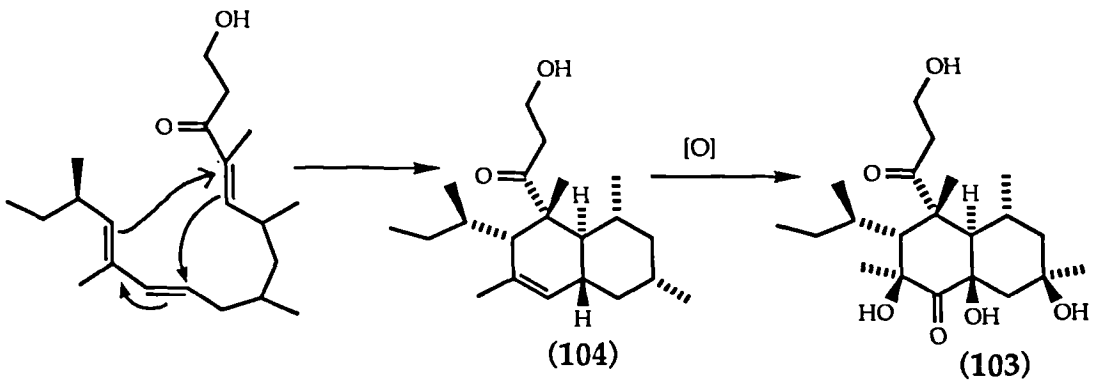
(99)

This inhibitor blocks the conversion of kaurene (100) to kaurenol (101) on the gibberellin (eg gibberellic acid, 102) biosynthetic pathway,[61] scheme 2.5.2



Scheme 2.5.2: The oxidation of kaurene (100) to kaurenol (101) on the biogenetic pathway to gibberellic acid (102), a step inhibited by ancymidol (99).

This inhibitor has been applied to the study of plant metabolism,[62] but was also employed in the study of betaenone B (103) biogenesis, a polyketide metabolite produced by the fungus *Phome betæ*. The biosynthesis of betaenone B (104) had already been shown to be the result of the sequential condensation of acetate with eight malonate units, with branching methylation resulting from methionine.[63] The use of [1-^{13}C, $^{18}O_2$]acetate indicated that the keto oxygen on C-1 was not acetate derived. From this a biogenetic hypothesis invoking an intra-moleculer Diels-Alder reaction was postulated,[64] as shown in scheme 2.5.3.



Scheme 2.5.3: The biosynthesis of betaenone B (103)

Experiments using ancymidol (99) as a mono-oxygenase inhibitor led to the suppression of the production betaenone B (103), the reduction in titre being directly proportional to the amount of ancymidol (99) administered. A new metabolite was found to accumulate, which was shown to be the intermediate (104) proposed as the Diels-Alder product.[64]

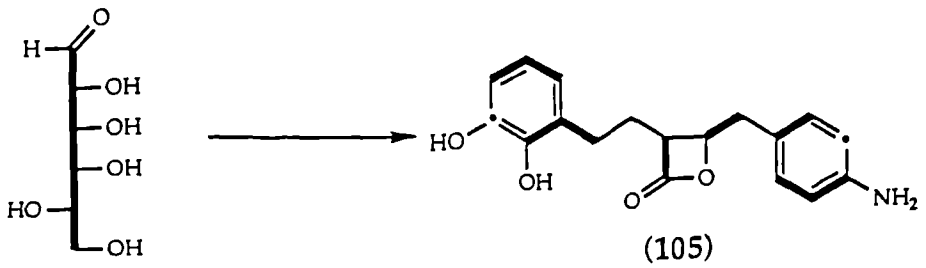
A similar experiment was conducted to measure the effect of ancymidol (99) on pseudomonic acid (44) biosynthesis. Thus to one of five flasks of secondary medium inoculated with the high producing strain of *Pseudomonas fluorescens* was added 0mg, 5mg, 10mg, 20mg, 40mg of ancymidol (99) at the commencement of metabolite production. The cultures were left to grow as usual before HPLC analysis. No significant difference in metabolite titre was found across the range of flasks, and no new peaks were observed by HPLC.

2.6: Conclusions and further work.

These results imply that 3-hydroxypropanoic acid (75) is not intimately involved in the biosynthesis of pseudomonic acid (44). However, the incorporation of label from the singly labelled form needs to be clarified. The original hypothesis regarding the involvement of β-hydroxy-β-methyl glutarate (64) in pseudomonic acid (44) biosynthesis invoked 5-hydroxypentanoic acid as an intermediate (scheme 2.2.5). The possibility of this being a chain starter may be worth investigating.

The multiply labelled malonate (73) experiment showed the C-7'/C-8' bond is not constructed as a result of fatty acid'/polyketide type processes. A further experiment employing D-[U-^{13}C]-glucose (10) may lead to further information regarding the biosynthetic origin of this bond. A recent example of the value of such an approach is in the elucidation of the biosynthesis of obafluorin (105), a β-lactone metabolite from *Ps. fluorescens* (ATCC 39502).

In order to define the separate units involved in obafluorin biogenesis D-[U-^{13}C]-glucose (10) was fed to *Ps. fluorescens* and the isolated metabolite analysed by ^{13}C nmr spectroscopy.[65] The resultant ^{13}C-^{13}C couplings (scheme 2.6.1) showed the intact bond incorporations, and demonstrated that the aromatic rings were of shikimate (36) origin. Two intact C_2 units were also involved, and one of these was later shown to be derived from glycine, via glyoxylate (17).[66]



(10) (105)

Scheme 2.6.1: Result form feeding D-[U-^{13}C]-glucose in obafluorin (**105**) biosynthesis

Another possibility is from label being introduced as a result of the decarboxylation of 'acids' and subsequent metamorphosis to a reduced state before (or during) incorporation into C-7'. The carboxyl groups of acetate, propionate and (possibly) 3-hydroxypropionate all label this position. It would be interesting to discover whether any other simple acids exhibit similar behaviour.

No positive information has been gained regarding the proposed biosynthesis of pseudomonic acid (**44**) from monic acid (**84**) and 9-hydroxynonanoic acid (**45**). Further information may be forthcoming from the genetic/mutant strain work. Repetition of the ancymidol (**99**) inhibition experiment with the normal producing strain of *Ps. fluorescens* may be worthwhile, as it is possible that the high producing strain effectively swamped any inhibition.

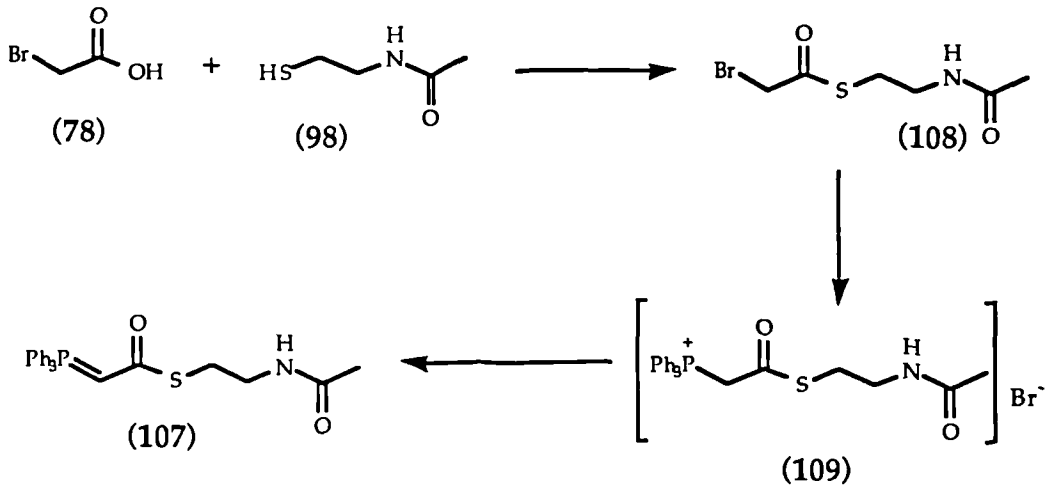
The experiments described earlier in the chapter regarding the use of labelled 9-hydroxynonanoic acid (**45**) gave results consistent with complete catabolism. However, one problem with these experiments may be due to a lack of sensitivity in using a singly labelled compound. This may be overcome by the use of a doubly (bond) labelled precursor, in the form of the N-acetylcysteamine thioester (**106**). Some work towards this has been undertaken.

Studies to produce the N-acetylcysteamine thioester of 9-hydroxy nonanoic acid (**106**) directly from the acid and the thiol using the DCC/DMAP[53] or $PhOP(O)Cl_2$/Py[67]coupling methods (to be further discussed in chapter three) were low yielding. As a doubly ^{13}C labelled compound was required, however, a different approach was considered.

As catabolism has been shown to be the dominant pathway the labels needed to be present in a manner such that any intact incorporation could be observed. This is possible if the labelled bond is equivalent to one newly

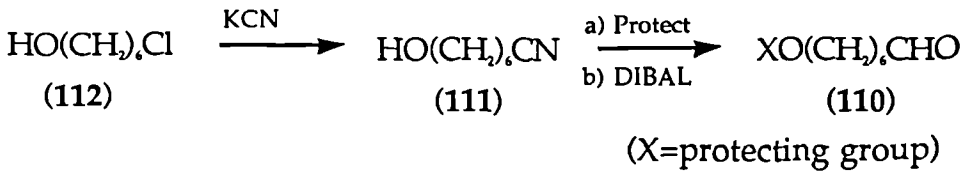
formed by an acetate/malonate condensation, as opposed to one that is present within the condensing moiety. The target molecule therefore chosen was the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-9-hydroxy nonanoate (**106**). The primary sources of labels from this scheme are [2-^{13}C]-bromoacetic acid (**78**) and potassium [^{13}C]-cyanide, and these are proposed to be coupled by a Wittig reaction.[68]

For this, the synthesis of the required ylid (**107**) was achieved by the coupling of bromoacetic acid (**78**) with N-acetylcysteamine (**96**) using DCC/DMAP,[53] followed by reaction of the resultant thioester (**108**) with triphenylphosphine to produce the N-acetylcysteamine thioester of [(triphenylphosphoniumacetate) bromide] (**109**). Titration of this against sodium hydroxide, with phenolphthalein indicator, produced the required ylid (**107**) in 46% overall yield, scheme 2.6.2.



Scheme 2.6.2: Synthesis of the ylid (**107**)

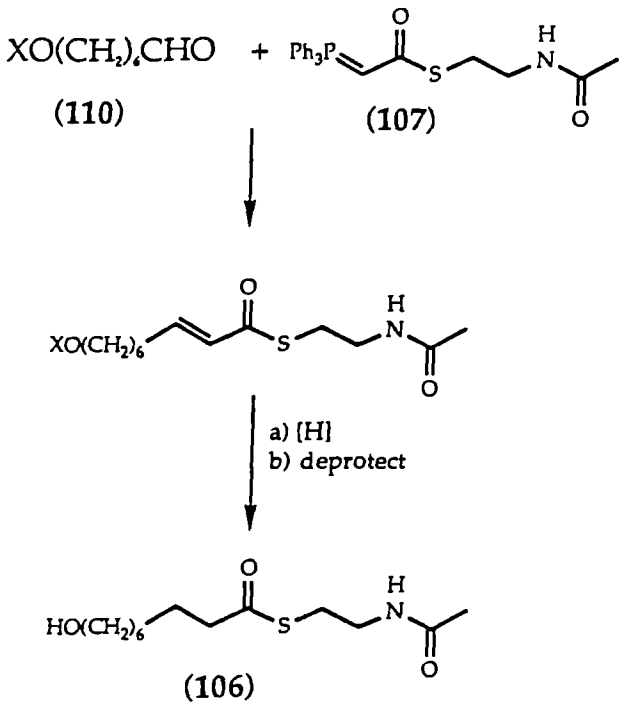
For the second half of the coupling reaction, the aldehyde (**110**) was required. Thus, 7-hydroxypropionitrile (**111**) was synthesised in 92% yield by the action of cyanide on 6-bromohexan-1-ol (**112**).



Scheme 2.6.3: Synthesis of the aldehyde (**110**)

Ethoxyethoxy ether protection of this in quantitative yield proceeded reduction of the nitrile moiety to the required aldehyde (110) by DIBAL[69] in 76% yield, scheme 2.6.3

Preliminary investigations into the Wittig coupling, scheme 2.6.4, indicate that the reaction proceeds under refluxing chloroform conditions. However, separation from the triphenylphosphine oxide by-product has not yet been achieved. Further work towards optimisation of this synthesis is required, particularly with respect to the choice of protection group, the DIBAL reduction, the coupling reaction, and also the final hydrogenation step, shown in scheme 2.6.4.



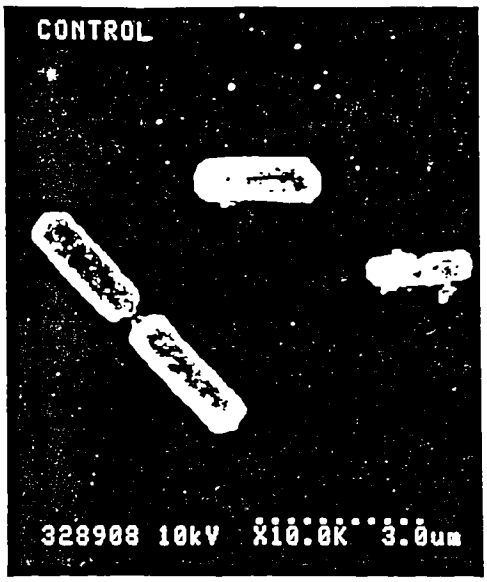
Scheme 2.6.4: Wittig coupling of aldehyde (107) with ylid (104), prior to hydrogenation to produce the N-acetylcysteamine thioester of 9-hydroxynonanoate.

APPENDIX 2.A.1

Electron microscopy studies on Ps. fluorescens after cell lysis experiments

These studies were undertaken after completion of the cell free investigations into the biogenesis of pseudomonic acid (44), but the samples examined were taken from those used in the experiments.

The accompanying photographs clearly show that sonication treatment had the most dramatic effect upon the cells (figure 2.A.1), when compared to an untreated cell colony. The attempt at liquid shear cell rupture by passage through a French press appears to have had some effect, as shown by the resultant debris. However, large numbers of intact cells are still present.



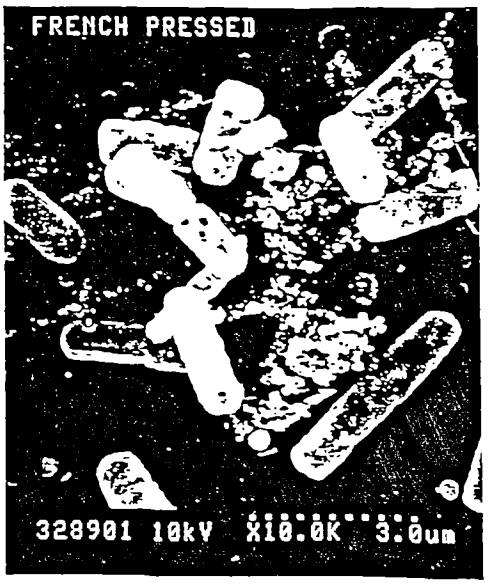


Figure 2.A.1: Electron microscopy studies on lysis of *Ps. fluorescens* by means of sonication and French press shear.

The use of lysozyme by itself led to no discernable change in the cell colony (figure 2.A.2). Treatment with EDTA prior to lysozyme treatment led to apparent shrinkage of the cells, but there is no evidence for cell lysis.

Therefore, it seems that sonication is the method of choice for further studies of this kind.

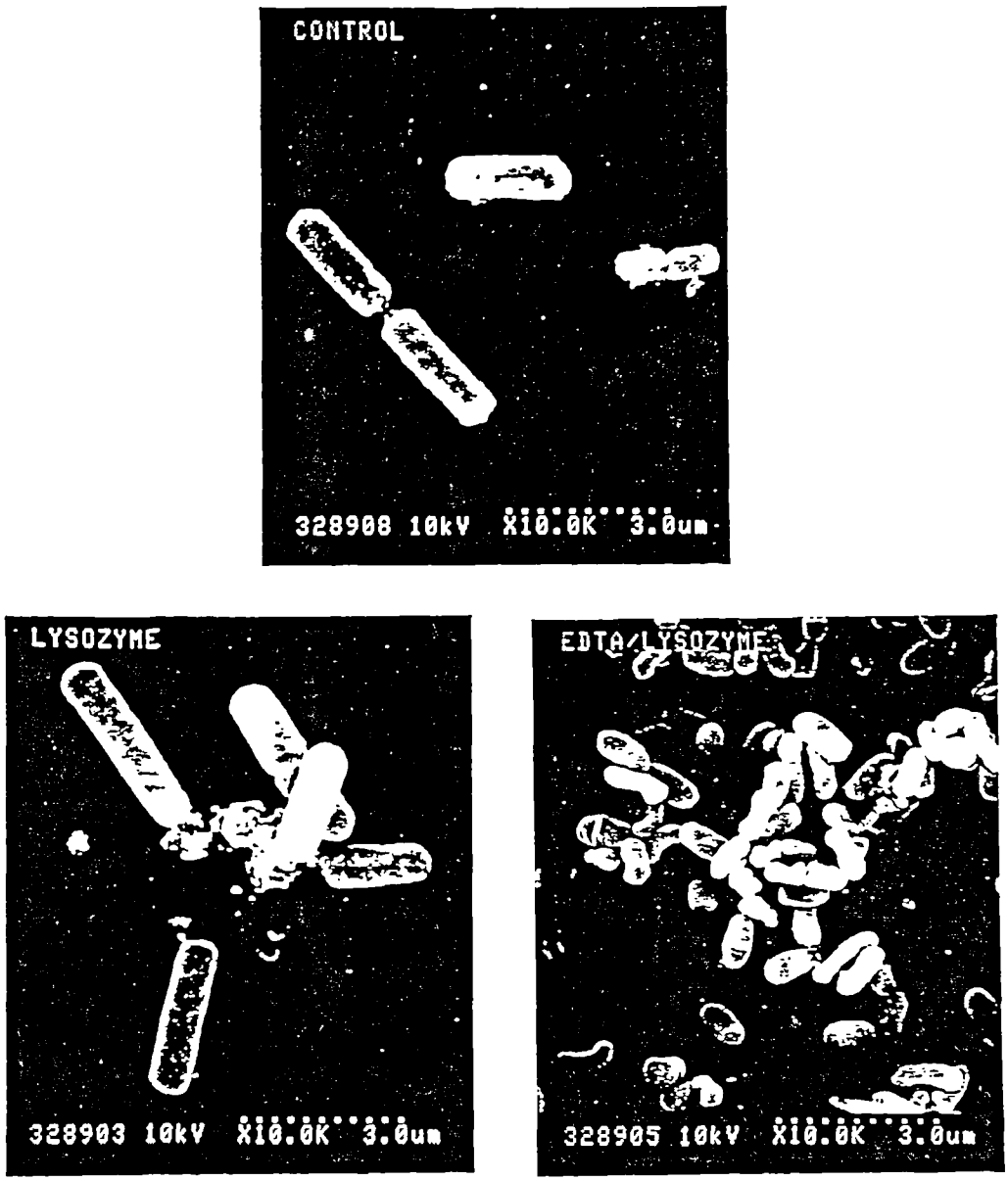


Figure 2.A.2: Electron microscopy studies on lysis of *Ps. fluorescens* by means of lysozyme and EDTA/lysozyme.

EXPERIMENTAL

General experimental

All ^{1}H and ^{13}C nmr spectra were recorded on a Jeol GX270 and PMX60 instruments in $C^{2}HCl_3$ solution and were referenced to tetramethyl silane, except those stated to have been run in $^{2}H_2O$ which were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

Melting points were determined on a Kofler hot-stage apparatus, and were uncorrected.

Mass spectra were obtained from a Kratos MS9 spectrometer with VG ZAB ion source and electronics.

Scintillation counting was performed on a LKB 1215 Rackbeta scintillation counter, with unisolve E as the scintillant.

Infra-red spectra were recorded on a Perkin-Elmer 881 spectrometer.

Gas chromatography was carried out using a Perkin-Elmer 8320 capillary gas chromatograph. High performance liquid chromatography was performed on a Gilson 303 system. The HPLC column was 250mm in length, with internal diameter 4.1mm and Alltech versapack C-18 10U packing.

Preparative thin layer chromatography was carried out on 200 x 200mm glass plates coated with silica gel (0.25, 0.5 or 1.0 cm, Fluka 60765 Kieselgel Gf245). Bands were visualised by the use of ultra-violet light (245nm) or via staining with ammonium molybdate (10% w/v in 2M sulphuric acid). Flash column chromatography was carried out using silica gel (40-63μm, Kieselgel 60, Merck, and 220-440 mesh, Kieselgel 60, Fluka).

Microbiological work was performed under sterile conditions, in a Biomat Class II microbiological cabinet. Sterilisation of all materials prior and subsequent to use was carried out in an autoclave under the described conditions.

Sonication was carried out on an MSE Soniprep 150 machine with a 9.5mm probe. The French press was an SLM Aminco FA079 press with an 20K manual fill FA073 cell. Lysozyme was purchased from BDH chemicals Ltd. and had 25,000 units mg^{-1}.

Buffer solutions were made using tris-HCl.

All solvents were dried and distilled prior to use according to published procedures.[70] Nitrogen was dried by passage through a silica gel/calcium chloride column.

Rehydration of *Ps. fluorescens*

A freshly opened lyophile of *Ps. fluorescens* was rehydrated by suspension in 0.5ml of sterile nutrient broth. The suspension was deposited in 4.5ml of sterile nutrient broth with stirring to ensure homogeneity. 10µl Loopfuls of this suspension were streaked onto 1/10 6sp agar slopes, the constitution of which is shown in table 2.E.1. These were left to grow at 25°C for 48 hours prior to storage at 4°C, under which conditions they were viable for four months.

Culturing of *Ps. fluorescens.*

A slope was flooded with 5ml of sterile nutrient broth and the bacteria suspended in this. A 1ml aliquot of this suspension was transferred to a sterile 250ml conical flask containing 25ml of primary medium, the constitution of which is shown in table 2.E.2. The inoculated flask was then shaken at 240 rpm for 24 hours at 22-24°C.

After this time ten 250ml conical flasks each containing 25ml of secondary medium (the constituents of which are shown in table 2.E.3) were each inoculated with a 1ml aliquot of the primary medium. These flasks were then shaken at 240 rpm for 28 hours at 22-24°C.

These procedures were used for the culturing of both the NCIB 10586 and PF3/N/2 strains of *Ps. fluorescens.*

Yeatex	0.285%
Glucose	0.11%
Na_2HPO_4	0.26%
KH_2PO_4	0.24%
$(NH4)_2SO_4$	0.5%
Agar	3.0%

Medium adjusted to pH 7.0 prior to sterilisation (121°C, 15psi)

Table 2.E.1 : Constituents of medium for 1/10 6sp agar slopes

Yeatex	2.85%
Glucose	0.11%
Na_2HPO_4	0.26%
KH_2PO_4	0.24%
$(NH_4)_2SO_4$	0.5%

Medium adjusted to pH 7.0 prior to sterilisation (121°C, 15psi)

Table 2.E.2: Constituents of primary (growth) medium.

Arkasoy-50	2.0%
Cornsteep liquor	0.5%
$(NH_4)_2SO_4$	0.5%
$MgSO_4.7H_2O$	0.05%
Na_2HPO_4	0.1%
KH_2PO_4	0.15%
KCl	0.1%
CaCO3	0.625%
Glucose	6.0%
Antifoam L-81	3 drops

Non-essential additives were : $MnCl_2.4H_2O$ (1ml of a 2% solution l^{-1})
$CoCl_2.6H_2O$ (1ml of a 0.03% solution l^{-1}).

Medium adjusted to pH 7.0 prior to sterilisation (115°C, 10psi)

Table 2.E.3: Constituents of secondary (production) medium.

Isolation of methyl pseudomonate (58)

The combined secondary media were centrifuged (10,000g, 4°C, 10 minutes) and the supernatant was acidified to pH 4.5, saturated with sodium chloride and extracted with ethyl acetate (5 x 150ml). The combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The orange residue was taken up in 5ml of methanol and treated with an excess of ethereal diazomethane, in a vessel devoid of ground glass joints. After two hours the diazomethane was blown off with nitrogen and the mixture concentrated *in vacuo*. Purification by prep. TLC (2.5% methanol in ethyl acetate) yielded **methyl**

pseudomonate (58) as a colourless oil which solidified upon refrigeration. υ_{max} 3309, 1742, 1717, 1651cm^{-1}; δ_H 0.94 (3H, d, J=7.4Hz, **H-17**), 1.22 (3H, d, J=6.5Hz, **H-14**), 1.31-1.38 (9H, br s, **H-4', H-5', H-6', H-12**), 1.44-1.98 (6H, m, **H-3', H-8', H-9**), 2.03 (1H, m, **H-8**), 2.22 (4H, s, **H-4ax, H-15**), 2.31 (2H, t, J=7.6Hz, **H-2'**), 2.46, 2.64 (1H, m, **H-4eq**), 2.68-2.73 (1H, m, **H-11**), 2.81 (1H, m, **H-10**), 3.46-3.59 (2H, m, **H-16eq, H-6**), 3.67 (3H, s, **OMe**), 3.94-3.74 (4H, m, **H-7, H-16ax, H-13, H-5**), 4.07 (2H, t, J=6.6Hz, **H-9'**), 5.76 (1H, s, **H-2**); δ_C 12.76 (**C-17**), 19.05 (**C-15**), 20.8 (**C-14**), 24.92 (**C-3'**), 25.97 (**C-7'**), 28.67 (**C-8'**), 29.05 and 29.11 (**C-4', 5', 6'**), 31.56 (**C-9**), 34.10 (**C-2'**), 39.53 (**C-8**), 42.86 (**C-12, 4**), 51.53 (OCH_3), 55.59 (**C-10**), 61.37 (**C-11**), 63.85 (**C-9'**), 65.34 (**C-16**), 69.02 (**C-6**), 70.42 (**C-7**), 71.46 (**C-13**), 74.88 (**C-5**), 117.69 (**C-2**), 156.66 (**C-3**), 166.77 (**C-1**), 174.44 (**C-1'**); m/z: 515 (M^++1, 0.7%), 514 (M^+, 0.8), 270 (48.6), 227 (87.3), 209 (34.4), 141 (44.6), 111 (92.3), 95.1 (44.6), 83 (51.4), 82 (100), 69 (60.9), 55 (71.2), 43 (65.4).

Growth production study

A 1ml aliquot was taken from the secondary medium every two hours, commencing 12 hours after inoculation. These were micro-centrifuged before analysis by HPLC, with a solvent consisting of methanol/0.05M ammonium acetate (3:2 at pH 4.5). The flow rate was 1.5ml min^{-1} with peaks being monitored at 230nm.

Preparation of ethereal diazomethane

For this preparation a self-contained distillation apparatus was used which contained no ground glass joints.

To 0.47g of potassium hydroxide was added 15ml of ethanol and 30ml of ether. The mixture was gently warmed until the ether began to slowly distil over into an ice-water cooled receptacle. At this point 1.79g of diazald was carefully added in 15ml of ether. The mixture was monitored such that a gentle reflux led to slow distillation of the yellow distillate. Reaction was complete when the distillate became colourless. The resultant solution was used immediately.

Synthesis of 9-hydroxynonanoic acid (45)

a) To 0.993g (4.75mmol) of 8-bromooctan-1-ol (85) in 5ml of methanol was added 0.974g (14.76mmol) of potassium cyanide in 5ml of water. The mixture was heated under reflux for 7 hours. After cooling the methanol was removed *in vacuo* and the residue extracted with ethyl acetate (3 x 10ml). The organic extracts were combined, dried over anhydrous magnesium sulphate and concentrated to give **9-hydroxynonanitrile (86)** (0.661g, 3.94mmol, 83%) as a pale yellow oil. υ_{max}(thin film) 3313, 2247 cm^{-1}; δ_H 1-2 (12H, br m, 6 x CH_2), 2.3 (2H, t, J=5.3 Hz, CH_2CN), 3.6 (2H, t, J=5.3 Hz, CH_2OH).

b) 9-Hydroxynonanitrile (86) (0.609g, 3.93mmol) was dissolved in 5ml methanol and to the solution was added 6ml of 2M NaOH solution. The mixture was heated under reflux overnight. After cooling the solution was extracted with ethyl acetate (10ml) and the aqueous layer then adjusted to pH 1.0 before re-extraction with ethyl acetate (5 x 100ml). The acidic extracts were combined, dried over anhydrous magnesium sulphate and concentrated to give **9-hydroxynonanoic acid (45)** (0.621g, 3.57mmol, 91%) as a white waxy solid. υ_{max}(nujol) 3298, 1732 cm^{-1}; δ_H 1-2 (12H, br m, 6 x CH_2), 2.2 (2H, t, J=5.6 Hz, CH_2CO_2H), 3.6 (2H, t, J=5.6 Hz, CH_2OH), 5.0 (2H, br s, 2 x OH); δ_C 24.58, 25.57, 28.49, 28.97, 33.98 (**C-2,3,4,5,6,7,8**), 62.81 (**C-9**), 174.12 (**C-1**); m/z 175 (M^++1, 1%), 174 (M^+, 1.1%), 157 (8%), 136 (6%), 69 (41%), 55 (100).

Synthesis of [1-^{13}C]-9-hydroxynonanoic acid (45)

a) To 0.502g (4.98mmol) of 8-bromooctan-1-ol (85) in 5ml of methanol was added 0.5g of potassium [^{13}C]-cyanide (7.46mmol) in 5ml of water. The mixture was heated under reflux for 7 hours. After cooling the methanol was removed *in vacuo* and the residue extracted with ethyl acetate (3 x 10ml). The organic extracts were combined, dried over anhydrous magnesium sulphate and concentrated to give **[1-^{13}C]-9-hydroxynonanitrile (86)** (0.627g, 4.03mmol, 81%) as a pale yellow oil. υ_{max}(thin film) 3309, 2245 cm^{-1}; δ_H 1-2 (12H, br m, 6 x CH_2), 2.3 (2H, dt, J_{CH}≈6.3 Hz J_{HH}=5.3Hz $CH_2{}^{13}CO_2H$), 3.6 (2H, t, J=5.3Hz, CH_2OH).

b): [1-^{13}C]-9-Hydroxynonanitrile (86) (0.627g 4.03mmol) was dissolved in 5ml methanol and to the solution was added 6ml of 2M NaOH solution. The mixture was heated under reflux overnight. After cooling the solution was extracted with ethyl acetate (10ml) and the aqueous layer then adjusted to pH 1.0 before re-extraction with ethyl acetate (5 x 100ml). The acidic extracts were combined, dried over anhydrous magnesium sulphate and concentrated to give **[1-^{13}C]-9-hydroxynonanoic acid (45)** (0.614g, 3.51mmol, 87%) as a white waxy solid. υ_{max} (nujol) 3302, 1731 cm^{-1}; δ_H 1-2 (12H, br m, 6 x CH_2), 2.3 (2H, m, $CH_2{}^{13}CO_2H$), 3.6 (2H, t, J= 5.6 Hz, CH_2OH), 4.8 (2H, br s, 2 x OH); δ_C 174.15; m/z 176 (M^++1, 0.5%), 175 (M^+, 1%), 158 (8.6), 139 (6.5), 69 (43), 55 (100).

Saponification of methyl pseudomonate (58)

To methyl pseudomonate in 3ml of triethyl orthoformate was added a crystal of PTSA. After 30 minutes the solvent was removed *in vacuo* and 10ml of 2ml NaOH added to the residue. This was stirred at 65°C for three hours, cooled and methanol added. The pH was adjusted to 1.5 for 15 minutes, then to 9.0 at which it was maintained for three hours. The pH was further adjusted to 3.0, and the mixture extracted with ethyl acetate (5 x 30ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The residue was taken up in 8ml of methanol and treated with an excess of ethereal diazomethane for 5 hours. The solvents were removed and the mixture purified by preparative TLC (dichloromethane/acetone, 1:1 with 4% methanol). The band at Rf 0.9 corresponded to **methyl 9-hydroxynonanoate (88)**, υ_{max} (thin film) 3367, 1651 cm^{-1}; δ_H 1-2 (12H, br m, 6 x CH_2), 2.31 (2H, t, J=5.7Hz, CH_2CO_2Me), 3.62 (2H, t, J=5.9Hz, CH_2OH), 3.67 (3H, s, OCH_3), and that at Rf 0.55 was **methyl monate (87)**. υ_{max}($CHCl_3$) 3297, 1711, 1647 cm^{-1}; δ_H 0.92 (3H, d, J=7.1Hz, CH_3), 1.18 (3H, d, J=6.6Hz, CH_3), 1.4 (1H, m, **H-12**), 2.05 (1H, m, **H-8**), 2.18 (3H, s, **H-15**), 2.5-2.75 (3H, m, **H-10, H-11, H-4**), 3.45-3.75 (6H, m, **H-5, H-6, H-7, H-13, H-16**), 3.65 (3H, s, OCH_3), 5.75 (1H, s, **2-H**)]

Synthesis of disodium malonate

a) To 1g (12.2 mmol) of anhydrous sodium acetate was added 5ml of triethyl phosphate, and the mixture heated to reflux for 3 hours. After cooling the condenser was linked to a vacuum pump via two traps. The

first of these was cooled with ice/salt, whilst the second was cooled with liquid nitrogen. The system was evacuated and the reaction mixture gently warmed such that ethyl acetate collected in the second trap, whilst any triethyl phosphate collected in the first trap. This yielded 0.89g (10.1 mmol, 83%) of **ethyl acetate** as a colourless liquid. δ_H 1.25 (3H, t, J=8Hz, CH_3), 2.05 (3H, s, CH_3), 4.18 (2H ,q, J=8Hz, OCH_2)

b) To 20ml of freshly distilled anhydrous THF at -78°C under an atmosphere of dry nitrogen was added of hexamethyldisilazide (2.113g, 13.13mmol). To this was added 5.08ml of butyl lithium as a 1.6M solution in hexanes (12.69mmol). After 30 minutes ethyl acetate (0.787g, 8.75mmol) was added dropwise as a solution in THF. After a further half hour ethyl chloroformate (1.425g, 13.13mmol) was added in THF. The mixture was left for 2.5 hours prior to addition of 10ml of 1M HCl. The layers were separated and the aqueous layer extracted with ether (2 x 20ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield an orange residue. This was purified by bulb to bulb distillation (66°C, 6mmHg) to yield 0.994g (6.21mmol, 71%) of **diethyl malonate (73)**. υ_{max}(thin film) 2996, 1731cm^{-1}; δ_H 1.25 (6H, t, J=6Hz, CH_3), 3.35(2H, s, CH_2), 4.18 (4H, q, J=6Hz, CH_2)

c): To the diethyl malonate (73) (0.842g, 5.27mmol) was added 5.22ml of 2.07M NaOH solution. The mixture was stirred overnight. The water was removed *in vacuo* prior to freeze drying. This gave g (0.78g, 5.27mmol, 100%) of **disodium malonate** as a white solid.υ_{max}(nujol) 1562, 1455, 1411 cm^{-1}; $\delta_H(^2H_2O)$ 3.12 (2H, s, CH_2)

Synthesis of disodium [1,2-$^{13}C_2$]-malonate

a): To 1g (11.9 mmol) of anhydrous sodium [1,2-$^{13}C_2$]-acetate was added 5ml of triethyl phosphate, and the mixture heated to reflux for 3 hours. After cooling the condenser was linked to a vacuum pump via two traps. The first of these was cooled with ice/salt, whilst the second was cooled with liquid nitrogen. The system was evacuated and the reaction mixture gently warmed such that ethyl acetate collected in the second trap, whilst any triethyl phosphate collected in the first trap. This yielded 0.833g (9.28

mmol, 78%) of **ethyl [1,2-$^{13}C_2$]-acetate** as a colourless liquid. δ_H 1.25 (3H, t, J=8Hz, CH_3), 2.05 (3H, dd, $^1J_{CH}$=129Hz, $^2J_{CH}$=7Hz, $^{13}CH_3$), 4.18 (2H, dq, J_{HH}=8Hz, $^3J_{CH}$=2Hz, OCH_2)

b) To 20ml of freshly distilled anhydrous THF at -78°C under an atmosphere of dry nitrogen was added 2.208g (13.69mmol) of hexamethyldisilazide. To this was added 5.29ml of butyl lithium as a 1.6M solution (13.23mmol) in hexanes. After 30 minutes ethyl [1,2-$^{13}C_2$]-acetate (0.821g, 9.12mmol) was added dropwise as a solution in THF. After a further half hour ethyl chloroformate (1.425g, 13.13mmol) was added in THF. The mixture was left for 2.5 hours prior to addition of 6M HCL, water. The layers were separated and the aqueous layer extracted with ether (2 x 20ml)). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield an orange residue. This was purified by bulb to bulb distillation (66°C, 6mmHg) to yield (1.043g, 6.29mmol, 69%) of **diethyl [1,2-$^{13}C_2$]-malonate (73)**. υ_{max}(thin film) 3001, 1733 cm^{-1}; δ_H 1.25 (6H, t, J=6Hz, CH_3), 3.35 (2H, dd, $^1J_{CH}$=130Hz, $^2J_{CH}$=6.7Hz, $^{13}CH_2$), 4.18 (4H, m, CH_2)

c) To the diethyl [1,2-$^{13}C_2$]-malonate (73) (0.983g, 5.94mmol) was added 5.9ml of 2.07M NaOH solution. The mixture was stirred overnight. The water was removed *in vacuo* prior to freeze drying. This gave 0.889g (5.93mmol, 100%) of **disodium [1,2-$^{13}C_2$]-malonate** as a white solid. υ_{max}(nujol) 1560, 1450, 1410cm^{-1}; δ_H (^2H_2O) 3.1 (2H, dd, $^1J_{CH}$=130Hz, $^2J_{CH}$=6.4Hz, $^{13}CH_2$).

Synthesis of sodium 3-hydroxypropionate (77)

a) To 2-chloroethanol (0.609g, 7.547mmol) was added potassium cyanide (1g, 14.93 mmol) in 8ml of water. The mixture was heated at 45-48°C for six hours. After this time the solvent was removed *in vacuo* and the solid residue extracted with dichloromethane (3 x 15ml). The combined extracts were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield **3-hydroxypropionitrile (83)** (0.4632g, 6.48mmol, 86%) as a colourless oil. υ_{max} (thin film) 3367, 2251 cm^{-1};

δ_H 2.6 (2H, t, J=5.5Hz, CH_2CN), 3.4 (1H, br s, OH), 3.85 (2H, t, J=5.5Hz, CH_2OH); δ_C 21.3 (CH_2), 54.43 (CH_2OH), 118.77 (CN).

b) To 3-hydroxypropionitrile (83) (0.4223g, 5.95mmol) was added 3.02ml of 2.07M NaOH solution (6.25mmol). The mixture was warmed to 75°C and left at this temperature overnight. After cooling the aqueous mixture was extracted with 10 ml of ethyl acetate. The water was removed *in vacuo* and the residue freeze-dried to leave **sodium 3-hydroxy propionate (77)** (0.673g, 5.97mmol, 100%) as a white solid. υ_{max}(nujol) 3623, 3299, 1523, 1461 cm^{-1}; $\delta_H(^2H_2O)$ 2.38 (2H, t, 5.5Hz, CH_2CO_2Na), 3.75 (2H, t, J=5.5Hz, CH_2OH); δ_C 40.81 (CH_2), 59.92 (CH_2), 180.71 (CO_2Na)

Synthesis of sodium [1-^{13}C]-3-hydroxypropionate

a) To 2-chloroethanol (0.401g, 4.97mmol)) was added potassium [^{13}C]-cyanide (0.5g, 7.46 mmol) in 8ml of water. The mixture was heated at 45-48°C for six hours. After this time the solvent was removed *in vacuo* and the solid residue extracted with dichloromethane (3 x 15ml). The combined extracts were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield **[1-^{13}C]-3-hydroxypropionitrile (83)** (0.274g, 3.83mmol, 77%) as a colourless oil. υ_{max}(thin film) 3371, 2250 cm^{-1}; δ_H 2.6(2H, dt, J_{HH}=5.5Hz, J_{CH}=6.5Hz, $CH_2{}^{13}CN$), 2.54 (1H, br s, OH), 3.85 (2H, m, CH_2OH)

b): To [1-^{13}C]-3-hydroxypropionitrile (83) (0.267g, 3.76mmol) was added 1.91ml of 2.07M NaOH solution (3.95mmol). The mixture was warmed to 75°C and left at this temperature overnight. After cooling the aqueous mixture was extracted with 10 ml of ethyl acetate. The water was removed *in vacuo* and the residue freeze-dried to leave **sodium [1-^{13}C]-3-hydroxypropionate (0.426g, 3.78mmol, 100%)** as a white solid. υ_{max}(nujol) 3625, 3299, 1525, 1461 cm^{-1}; $\delta_H(^2H_2O)$ 2.38 (2H, m, $CH_2{}^{13}CO2Na$), 3.75 (2H, m, 2H, CH_2OH); δ_C 180.75.

Synthesis of sodium [1-^{13}C, 2-$^{2}H_2$]-3-hydroxypropionate (77)

b): To [1-^{13}C]-3-hydroxypropionitrile (0.2613g, 3.22mmol) was added 2.05ml of 1.68M $NaO^{2}H$ solution (in $^{2}H_2O$) (3.41mmol). The mixture was warmed to 75°C and left at this temperature overnight. After cooling the aqueous mixture was extracted with 10 ml of ethyl acetate. The water was removed *in vacuo* and the residue freeze-dried to leave **sodium [1-^{13}C,$^{2}H_2$]-3-hydroxypropionate (77)** (0.2879g, 3.184mmol,99%) as a white solid. υ_{max}(nujol) 3627, 3301, 1525, 1461 cm^{-1}; δ_H($^{2}H_2O$) 3.74 (1H, br s, $HOCH_2C^{2}H_2$).

Synthesis of cyanoacetic acid (82)

To bromoacetic acid (78) (19.158g, 137.8mmol) in 100ml of water at 50°C was slowly added 7.73g (72.9mmol) of potassium carbonate. The mixture was cooled to 0°C and 7.149g (14.59mmol) of potassium cyanide was slowly added as a solution on 10ml of water. This was warmed to room temperature for 30 minutes, to reflux for 5 minutes then cooled to 0°C for a further 30 minutes. The mixture was the acidified to pH 2.0 and concentrated *in vacuo* to leave a white solid. This was taken up in 50ml of ethanol, filtered and the filtrate concentrated to leave **cyanoacetic acid (82)** as a white solid (9.297g, 109.4mmol, 80%). υ_{max} 2264 cm^{-1}; δ_H 3.65 (2H, s, CH_2), 6.05 (1H, br s, OH); m/z: 85 (M^+, 4.8%), 68 (15), 599 (7.3), 44 (100), 41 (75).

Attempted Stephen reduction of cyanoacetic acid (82)

Anhydrous tin (II) chloride (0.7506g, 3.95mmol) was suspended in 5ml of freshly distilled anhydrous ether under an inert atmosphere. Anhydrous hydrogen chloride gas was bubbled through this with stirring until the tin (II) chloride completely disappeared and two layers formed. Cyanoacetic acid (0.2239g, 2.63mmol) was added and a precipitate immediately formed. This was isolated by filtration and treated with warm water. The resultant mixture was extracted with ethyl acetate (3 x 10ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated to give a white solid.

The ^{1}H nmr spectrum of this showed a multitude of resonances, none of which corresponded to expected product.

The identical procedure was carried out on ethyl cyanoacetate, and again the white solid isolated showed no resemblance to expected product by ^{1}H nmr spectroscopy.

Synthesis of N-acetylcysteamine (98)

a): To a three necked flask at 0°C containing 17.04g (149.5mmol) of 2-aminoethanethiol hydrochloride in 125 ml of water was attached 2 dropping funnels an a pH probe. In one funnel was placed 100ml 11.2 M NaOH, and in the other 42.42 ml of acetic anhydride. The NaOH and acetic anhydride were added dropwise to the aqueous solution a rate such that a pH of 8.0 was maintained in the stirred reaction mixture. After addition of the acetic anhydride was complete the pH of the mixture was adjusted to 7.0 and the mixture left stirring at room temperature for an hour. After saturation with sodium chloride the aqueous solution was extracted with dichloromethane (2 x 20ml). The combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated to give **N,S-diacetylcysteamine** as a colourless oil (23.90g, 148.4mmol,99%). This solidified upon refrigeration. υ_{max} ($CHCl_3$) 1655, 1545 cm-1; δ_H 1.99 (3H, s, CH_3COS), 2.36 (3H, s, CH_3CON), 3.03 (2H, t, J=6.2Hz, CH_2S), 3.41 (2H, dt J=6.4, 6.1Hz, CH_2N), 6.53 (1H, br s, NH); δ_C 22.9 (CH_2N), 28.5 (CH_2S), 30.5 (CH_3COS), 39.2 (CH_3CON), 176.7 (CON), 195.8 (COS); m/z 161 (M^+, 0.9%), 119 (83%), 118 (21.7).

b) To 20ml of water was added 0.927g (5.73mmol) of N,S-diacetyl cysteamine followed by 0.759g (18.9mmol) of sodium hydroxide. The mixture was stirred at room temperature for 30 minutes prior to adjustment of the pH to 7.0. Saturation with sodium chloride was followed by extraction with dichloromethane (5 x 20ml).. The combined organic layers were dried over anhydrous magnesium sulphate, filtered and dried to give 0.5734g (4.81mmol, 84%) of **N-acetylcysteamine (98)** as colourless oil. This was used immediately. υ_{max} (thin film) 1641, 1525 cm^{-1}; δ_H 1.42 (1H, t, J=6.5Hz, SH), 2.02 (3H, s, CH_3CON), 2.67 (2H, dt, J=8.4, 6.6Hz, CH_2S), 3.42 (2H, dt, J=6.6Hz, J=6.4Hz, CH_2N), 6.33 (1H, br s, NH); δ_C 23.0 (CH_2N), 24.4 (CH_2S), 42.2 (CH_3), 170.5 (CON); m/z 119 (M^+, 14.3%), 60 (90.4).

Preparation of 'cell free' extracts

Ps fluorescens was cultured as previously described until production of pseudomonic acid (44) was shown to have commenced by HPLC.

At this point the cells were centrifuged (10,000g, 4°C, 10 minutes) and the supernatant removed. The cells were then washed with pH 7.0 buffer water and re-centrifuged. A further wash was carried out to leave ca. 2.1g of cells (wet weight) per 25 ml of secondary medium. These were resuspended in 20ml of pH 7.6 buffer. Further work was carried out with re-suspension in pH 7.2 and 6.9 buffers.

a) sonication

The cell suspension was cooled to 0°C and subjected to intense sound energy for two minutes (six x twenty second cycles) with stirring to ensure efficient heat dissipation between each cycle.

b) lysozyme

To the cell suspension was added lysozyme (10mg ml^{-1}) as a solution in buffer. This was left stirring for two hours before immediate use.

c) EDTA/lysozyme.

Prior to treatment with lysozyme the cells were treated with 30ml of a 0.2M solution of disodium EDTA in buffer. Treatment with lysozyme then proceeded as before.

d) French press

The buffered cell suspension was passed through the press at 0°C with internal pressure of 3500psi.

For membrane free extracts the treated suspension was micro-centrifuged and the supernatant retained.

The cell free preparations were used immediately except for anhydrous organic solvent reactions, when the extracts were freeze-dried overnight.

Cell free reactions.

Initial reactions were carried out in aqueous buffer at pH 7.6. Monic acid (84) (as a 10mg ml^{-1} solution in buffer) and 9-hydroxy nonanoic acid (45) (as a 20mg ml^{-1} solution in 1,4-dioxan) were added to the 'cell free enzyme' such that the total volume was 500µl, and the concentrations of the reactants was 5mmol and 15mmol respectively. The procedure was repeated with the N-acetylcysteamine thioester of monic acid (97) in place of monic acid (84).

Further reactions were carried out at pH 7.2 and 6.9. The concentrations of monic acid (84) (or the thioester (97)) and 9-hydroxy nonanoic acid (45) were also reduced to 2mmol and 10mmol respectively, with the total volume maintained at 500µl.

Cell free extracts from all lysis experiments were used, in both the presence and absence of cellular membrane. Reaction times of two, four and eighteen hours were investigated.

For reactions in 'anhydrous' organic media, the reactant concentrations were monic acid (84) (or the thioester (97)) at 2mmol and 9-hydroxynonanoic acid at 10mmol. Pseudomonic acid was used alone at 2mmol concentration. The total volume of solvent was 500µl, and 30mg of freshly lyopholised cell free extract was added. The solvents investigated were 1,4-dioxan, tetrahydrofuran and acetonitrile. The addition of 0.1% or 0.5% of water to the system was also studied.

Reaction times of two, four and eighteen hours were investigated.

For each set of reactions, control systems were also set-up containing the reactants under equivalent concentrations, but with no addition of cell free extract

HPLC was used to monitor the reactions as previously described. In order to assay for the production monic acid the solvent system used was methanol/0.05M ammonium acetate (5:1 at pH 4.5.). Under no conditions was any variance from control reactions observed.

Study of mutant strains of *Ps. fluorescens.*

The mutant strains were grown under previously described culture conditions. Subsequent to centrifugation (10,000g, 4°C, 10 minutes) the supernatant was assayed by HPLC, with the solvent being methanol/0.05M ammonium acetate of ratios from 3:2 to 7:1 at pH 4.5. No production of

pseudomonic acid (44), monic acid (84) nor any other unusual metabolite was observed.

Synthesis of the N-acetylcysteamine thioester of monic acid (97)

To 30ml of freshly distilled anhydrous THF under nitrogen was added 0.917g (2.67mmol) of monic acid (84) to give a turbid solution. To this was added 0.512g (4.31mmol) of freshly prepared N-acetylcysteamine (98) in 2ml of THF followed by DCC (0.577g, 2.80mmol) and DMAP(0.04g, 0.28mmol) in 5ml THF, and the reaction left stirring overnight. The mixture was filtered, and the filtrate washed with 25ml of saturated ammonium chloride, dried over anhydrous magnesium sulphate, filtered, concentrated and purified by flash column chromatography to give 0.2127g (0.48mmol, 18%) of the **N-acetylcysteamine thioester of monic acid as a colourless syrup (97).** $\upsilon_{max}(CHCl_3)$ 3671, 3156, 1794, 1664, 1617, 1557, 1543, 1518 cm^{-1}; δ_H 0.92 (3H, d, J=7.3Hz, CH_3), 1.11 (3H, d, J=6.5Hz, CH_3), 1.38 (1H, m, CH), 1.69 (2H, m, CH_2), 1.98 (3H, s, CH_3CON), 2.02 (1H, m, CH), 2.2 (4H, s, CH_3, CH), 2.6-2.84 (3H, m, 3 x CH), 3.04 (2H, m, CH_2S), 3,41 (2H, m, CH_2N), 3.5-3.96 (8H, m), 6.17 (1H, s, **H-2**), 6.27 (1H, br s, NH); δ_C 12.68 (**C-17**), 20.27 (**C-15**), 20.81 (**C-14**), 23.19 (CH_2N), 28.39 (CH_2S), 31.57 (**C-9**), 39.68 and 39.89 (**C-8**, $COCH_3$), 42.74 (**C-12, 4**), 55.54 (**C-10**), 61.27 (**C-11**), 65.35 (**C-16**), 68.75 (**C-6**), 70.33 (**C-7**), 71.35 (**C-13**), 74.78 (**C-5**), 124.11 (**C-2**), 155.51 (**C-3**), 170.72 (CON), 189.42 (COS); m/z 327 (M^+-118, 1.6%), 227 (14.9), 196 (12.3), 179 (6), 199 (12.8), 111 (40), 96 (24), 60 (82.3), 43 (97.6), 30 (100)

Synthesis of 7-(1'-ethoxy)ethoxyheptanal (110)

a) To 0.572g (3.16mmol) of 6-bromoheptan-1-ol (**112**) was added 0.2501g (3.79mmol) of potassium cyanide in 10ml of water. This was heated to reflux for 3.5 hours. After cooling the mixture was extracted with dichloromethane and the combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated to give **7-hydroxyheptanitrile (111)** as a colourless oil (0.3259g, 2.57mmol, 82%). υ_{max} (thin film) 3486, 2245 cm^{-1}; δ_H 1.3-1.75 (8H, m ,4 x CH_2) 2.38 (2H, t, J=6.7 Hz, CH_2CN), 3.61 (2H, t, J=6.7Hz, CH_2OH)

b) 7-Hydroxyheptanitrile (**111**) (0.3259g, 2.457mmol) was dissolved in 10ml of ethoxyethene. To this was added 1 drop of trifluoroacetic acid the mixture left to stir overnight. The volatiles were removed *in vacuo* to yield 0.5117g (2.57mmol, 100%) of **7-(1'-ethoxy)ethoxyheptanitrile** as a pale straw oil. δ_H 1.18 (3H, t, J=6.9Hz, CH_3CH_2O), 1.24 (3H, d, J=6.8 Hz, CH_3CH), 1.27-1.78 (10H, m), 2.37 (2H, t, J=6.8Hz, CH_2CN), 3.4-3.7 (4H, m, CH_2O and CH_2OH), 4.65 (1H, m, $OCH(CH_3)O$)

c) To 0.329g (1.66mmol) of 7-(1'-ethoxy)ethoxyheptanitrile in 5ml of anhydrous toluene at room temperature under an inert atmosphere was added dropwise 1.10ml of a 1.5M solution of DIBAL in toluene (1.66mmol). The mixture was left stirring overnight. to this was added 10ml of a 1:1 mixture of methanol/5M HCl . The layers were separated and the aqueous portion extracted with ethyl acetate (3 x 10ml). The combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated to leave **7-(1'-ethoxy)ethoxyheptanal (110)** as a straw coloured oil. υ_{max} (thin film) 1719 cm^{-1}; δ_H 1.15 (3H, t, J=6.8Hz, CH_3CH_2O), 1.3-1.8 (13H, m) 2.44 (2H, t, J= 6.6Hz, CH_2CHO), 3.3-3.7 (4H, m, CH_2O and CH_2OH), 4.67 (1H, q, J= 6.3 Hz, $OCH(CH_3)O$) 9.77 (1H, s, CHO)

Synthesis of the N-acetylcysteamine thioester of triphenylphosphorane acetate (107)

a) To 2.967g (21.36mmol) of bromoacetic acid (78) in 10ml of dichloromethane under a nitrogen atmosphere at 0°C was added 3.305g of freshly prepared N-acetylcysteamine (98) (27.7mmol) in 5ml of dichloro methane. To this mixture was added 4.823g (22.4 mmol) of DCC and 0.261g (2.136mmol) of DMAP in 10ml of dichloromethane. The system was left to warm to room temperature overnight. The resultant mixture was filtered and the filtrate washed with saturated ammonium chloride solution, dried over anhydrous magnesium sulphate, filtered, concentrated *in vacuo* and purified by flash column chromatography (acetone/dichloromethane 2:3) to yield the **N-acetylcysteamine thioester of bromoacetate (108)** as white solid (3.832g, 15.97mmol, 75%). Mpt 98-99.5°C; υ_{max}(nujol) 3306, 1732, 1642, 1548 cm^{-1}; δ_H 1,98 (3H, s, CH_3CON), 3.17 (2H, t, J=6.2Hz, CH_2S), 3.49 (2H, m, CH_2N), 4.04 (2H, s, $BrCH_2CO$), 6.02 (1H, br s, NH); δ_C 23.12 (CH_2N), 29.66 (CH_2S), 33.28 (CH_2Br), 39.00 (CH_3), 170.43 (CON),

193.01 (COS); m/z (241 M^++1, 0.5%), 239 (M^+-1, 0.4) 160 (8.5), 118 (70.2) 60 (35), 43 (62), 30 (100)

b) The N-acetylcysteamine thioester of bromoacetate (**108**) (3.82g, 15.97mmol) was suspended in 50ml of toluene to which was added 4.171g (15.97mmol) of triphenyl phosphine. This was left for 48 hours with occasional shaking. The mixture was filtered and the pale peach residue was dried *in vacuo* over phosphorus pentaoxide to leave 5.692g (11.34mmol, 71%) of the **N-acetylcysteamine thioester of (triphenyl phosphonium acetate)] bromide (109)**. δ_H 2.0 (3H, s, CH_3CO), 3.05 (2H, m, CH_2S), 3.42 (2H, m, CH_2N), 5.8 (2H, d, J=17Hz, CH_2P), 7.6-7.9 (15H, m, 3x **phenyl**), 8.45 (1H, br s, NH);' m/z: (M^+ absent), 278 (12.9), 276 (27.6), 119 (15.8), 72 (13.2), 60 (89.1), 43 (63.8), 30 (100)

c) The N-acetylcysteamine thioester of (triphenylphosphonium acetate)] bromide (**109**) (5.692g, 11.34mmol) was dissolved in water (50ml) and titrated against 0.16M NaOH with phenolphthalein indicator. The resultant mixture was extracted with dichloromethane (5 x 50ml) and the combined organic layers were dried over anhydrous magnesium sulphate, filtered, concentrated *in vacuo* and purified by flash column chromatography (acetone/dichloromethane, 2:3) to yield the **N-acetylcysteamine thioester of triphenylphosporane acetate (107)** as a colourless oil (4.535g, 10.77mmol, 95%). $\upsilon_{max}(CHCl_3)$ 3285, 1732, 1635, 1584, 1551 cm^{-1}; δ_H 1.69 (3H, s, CH_3CON), 2.99 (2H, m, CH_2S), 3.38 (2H, m, CH_2N), 3.83 (1H, br s, CHP), 7.4-7.75 (15H, m, 3 x **phenyl**); δ_C 23.17 (CH_2N), 27.56 (CH_2S), 42.98 (CH_3), 48.95 (d, J_{cp}=109.4Hz, HC=P), 125.51, 126.85, 129.06, 129.26, 132.26, 132.68, 133.01, 133.14 (all **aromatic C**), 170.56 (CON), 181.76 (COS); m/z 303 (M^+-118, 1.3%), 119 (19.5), 118 (13.0), 77 (16.7), 72 (18.0), 60 (79.5), 43 (75.1), 30 (100).

Incorporation studies

a) Sodium [2-^{13}C]-propionate

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing sodium [2-^{13}C]-propionate (250mg) was

prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 11mg of methyl pseudomonate (58) were isolated. The ^{1}H nmr spectrum was identical to that of authentic material.

b) [1-^{14}C]-9-Hydroxynonanoic acid (45)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing [1-^{14}C]-9-hydroxynonanoic acid (45) (250mg, 10.41μCi) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 34mg of methyl pseudomonate were isolated, with an activity of 0.82μCi. The ^{1}H nmr spectrum was identical to that of authentic material. Hydolysis of this as previously described gave methyl monate (87) and methyl 9-hydroxynonanoate (88), both of which contained significant amounts of activity.

c) [1-^{13}C]-9-Hydroxynonanoic acid (45)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing [1-^{13}C]-9-hydroxynonanoic acid (45) (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 21mg of methyl pseudomonate were isolated. The ^{1}H nmr spectrum was identical to that of authentic material.

d) Tetradecylthiopropionic acid (89)

Primary and secondary media were inoculated as previously described. A 5ml solution (DMSO) containing tetradecylthiopropionic acid (89) (30mg) was prepared. 2.5ml of this was added to the secondary medium (0.25ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 21mg of methyl pseudomonate were isolated. The ^{1}H and ^{13}C nmr spectra were identical to that of authentic material.

e) [1-^{13}C]-9-Hydroxynonanoic acid (45) plus tetradecylthiopropionic acid (89)

Primary and secondary media were inoculated as previously described. A 10ml solution (DMSO) containing [1-^{13}C]-9-hydroxynonanoic acid (45) (250mg) and tetradecylthiopropionic acid (89) (30mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 15mg of methyl pseudomonate were isolated. The ^{1}H nmr spectrum was identical to that of authentic material.

f) Disodium [2,3-$^{13}C_2$]-malonate (73)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing disodium [2,3-$^{13}C_2$]-malonate (73) (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 10mg of methyl pseudomonate were isolated. The ^{1}H nmr spectrum was identical to that of authentic material.

g) Sodium [1-^{13}C]-3-hydroxypropionate (77)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing sodium [1-^{13}C]-3-hydroxypropionate (77) (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 29mg of methyl pseudomonate were isolated. The ^{1}H nmr spectrum was identical to that of authentic material.

In a repeat experiment, 17mg of methyl pseusomonate were isolated. The ^{1}H and ^{13}C nmr spectra were identical to that of an authenic sample

h) Sodium [1-^{13}C, 2-$^{2}H_2$]-3-hydroxypropionate (77)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing sodium [1-^{13}C, 2-$^{2}H_2$]-3-hydroxypropionate (77) (250mg was prepared. 5ml of this was added to the

secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 13mg of methyl pseudomonate were isolated. The ^{1}H and ^{13}C nmr spectra were identical to that of an authenic sample.

In a repeat experiment, 18mg of methyl pseudomonate were isolated.The ^{1}H and ^{13}C nmr spectra were identical to that of an authenic sample.

i) Ancymidol (99)

Primary and secondary media were inoculated as usual with *Ps. fluorescens* PF3/N/2. To one of four flasks of secondary medium was added either 5, 10, 20 or 40mg of ancymidol (99) seventeen hours after inoculation. As a control, a fifth flask was inoculated, but no ancymidol (99) was added. After a further thirty-one hours the media were centrifuged (10,000g, 4°C, 10 minutes) and the supernatant assayed by HPLC under previously described conditions. No significant variation in titre was observed in any of the flasks with repect to the control.

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CHAPTER THREE

MONIC ACID

3.1: POLYKETIDE CHAIN ELONGATION IN PSEUDOMONIC ACID BIOSYNTHESIS

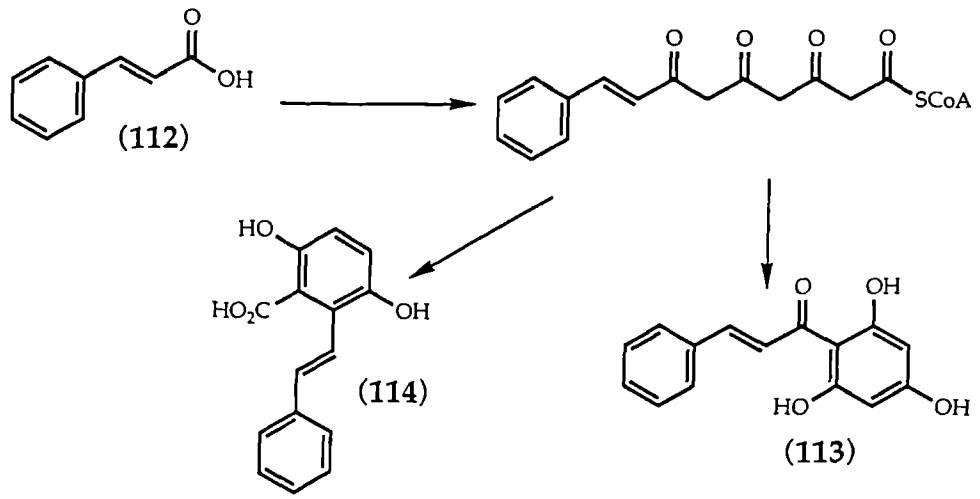
3.1.1: Introduction

Early work on the biogenetic origins of pseudomonic acid (44) indicated that the monic acid (84) moiety was polyketide in origin.[1] Methyl branching was shown to be the result of methylation by S-adenosyl methionine (59) for carbons 16 and 17. Carbon 15, however, was found to result from the terminal carbon of a cleaved acetate unit.

The first part of this chapter will discuss the polyketide biosynthetic pathway postulated to lead to pseudomonic acid (44). Consideration of the processes leading to the methylations is given in later sections.

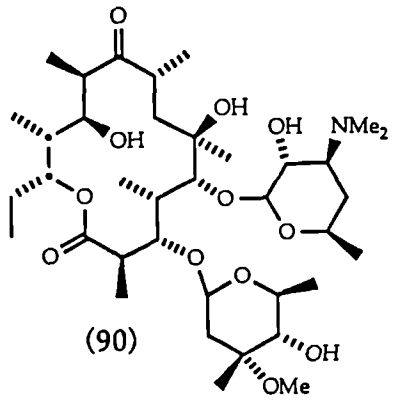
3.1.2: The classical polyketide hypothesis

The idea that many aromatic natural products were biosynthesised from the sequential condensation of acetate and malonate prior to chain modification operations was introduced in the first chapter. Further work in this field showed that structural variation could be introduced by the use of alternative chain starting or extending units.[2] In particular, variants of the starting unit were more prevalent. For example, cinnamic acid (112) was found to be the starting unit for chalcone (113) and stilbene (114) biosynthesis,[3] scheme 31.1.

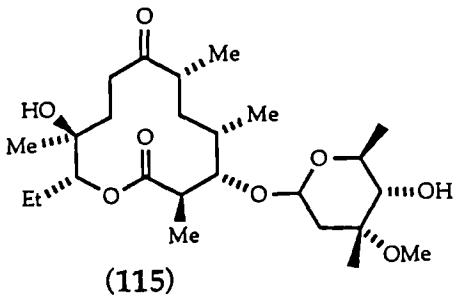


Scheme 3.1.1: Cinnamic acid (112) as a starter in polyketide biosynthesis

As information accumulated it was realised that the biosynthesis of many non-aromatic natural products could also be accounted for by a polyketide pathway. Many metabolites containing 1,3 oxidation patterns were known, although the actual oxidation levels varied. Despite the species restricted distribution of such metabolites, a common biogenetic pathway appeared to be in operation.

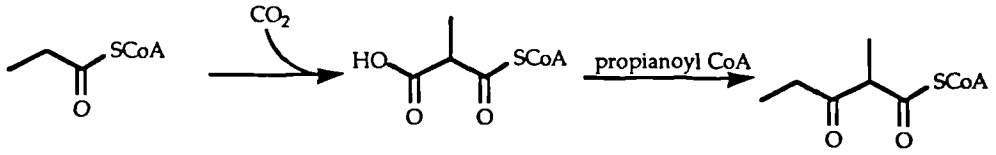


(90)



(115)

The macrolide lactones, eg erythromycin B (90) and methymycin (115), were shown to be the result of the sequential condensation of methylmalonate (derived from the α-carboxylation of propionate) onto simple propionate or butanoate starting units,[4] as shown in scheme 4.1.2.

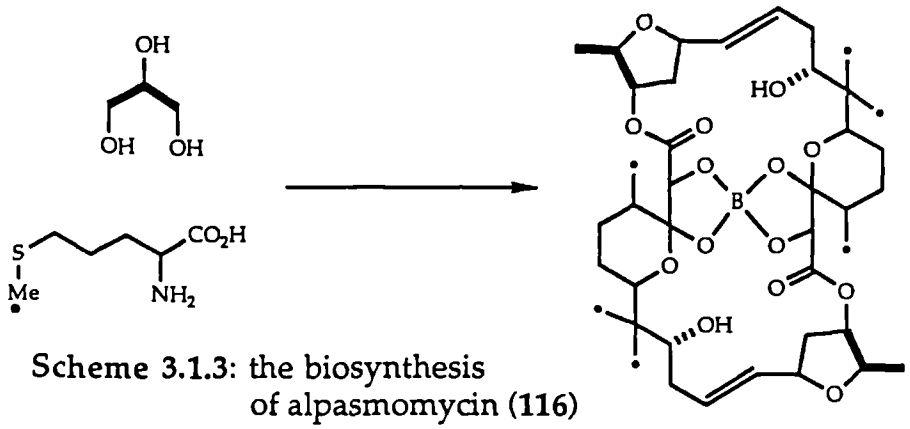


Scheme 3.1.2: Propionate as a starter and chain extender unit (as methylmalonyl CoA)

The use of these precursors introduced concomitant branching off the growing chain, thus eliminating any requirement for S-adenosyl methionine (59). An exception to this general rule is exhibited by aplasmomycin (116). This unusual boron containing macrolide produced by *Streptomyces griseus* was shown to be the result of sequential malonate condensations onto a glycerol derived C_3 starter,[5] scheme 3.1.3.

Unusual starting units were also discovered in non-macrolide polyketide derived metabolites. Coronatine (117), from *Pseudomonas syringæ* pv. *atropurpuræ* and *Pseudomonas syringæ* pv *glycinæ*, was

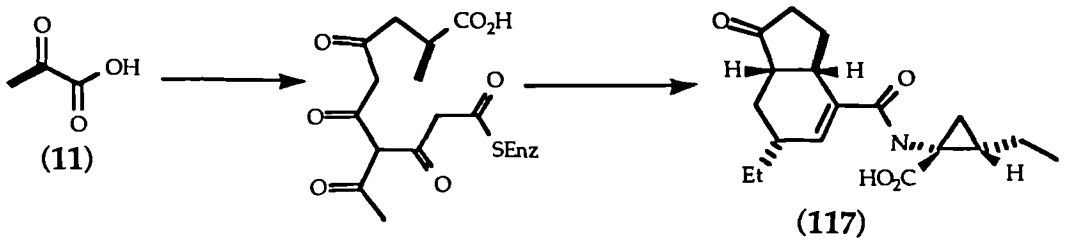
shown to be derived from the condensation of five malonate units onto a pyruvate starter,[6] scheme 3.1.4.



Scheme 3.1.3: the biosynthesis of alpasmomycin (116)

(116)

The classical ideas of polyketide aromatic biosynthesis seemed to apply to more complex metabolites. Experimental evidence began to emerge in the 1980's, however, that suggested the basic premise may be incorrect, leading to the formulation of a new polyketide hypothesis.



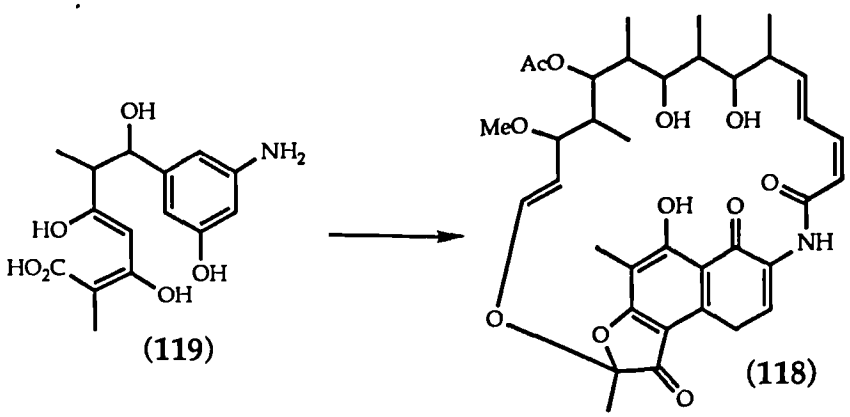
(11)

(117)

Scheme 3.1.4: The biosynthesis of coronatine (117)

3.1.3: Processive polyketide biosynthesis

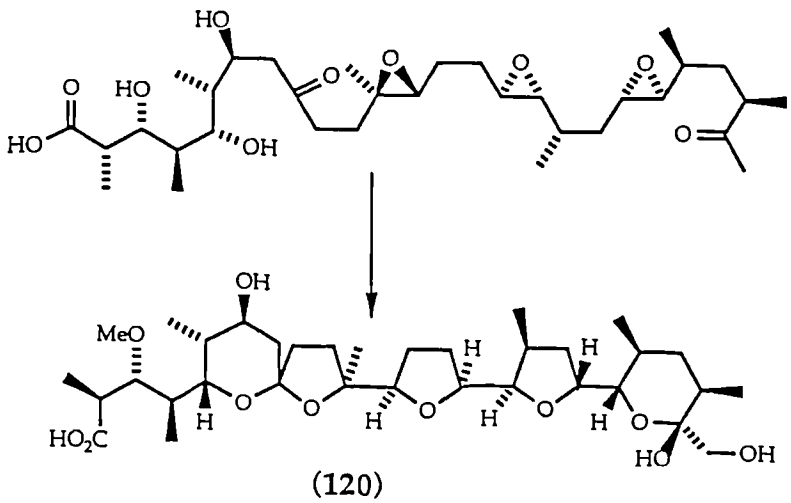
Rifamycin S (118) is a natural product produced by the organism *Norcardia mediterranei*.[7] Studies upon mutant strains of *N. mediterranei*, led to the isolation of metabolites that resembled part of the rifamycin structure.[8] The major isolate of these was shown to be 2,6-dimethyl-3,5,7-trihydroxy-7-(3'-amino-5'-hydroxyphenyl)-hepta-2,4-dienoic acid (119). As a result, it was deduced that these metabolites were biosynthetic precursors to rifamycin (118), scheme 3.1.5, and were the first compounds related to chain assembly processes to be isolated.



Scheme 3.1.5: A precusor to rifamycin (**118**) isolated from mutant strains of *Norcardia mediterranei*.

The polyether antibiotics,[9] for example monensin (**120**) and lysocellin (**121**), are a large class of natural products known to be of polyketide origin.[10] In 1985 Cane, Westley and Celmer postulated a unified stereo chemical and biogenetic model for this metabolic family,[11] following on from the unified stereochemical theory for the macrolide lactones formulated by Celmer in 1965.[12] Structural considerations of the polyether antibiotics, and in particular the basic biogenetic origins of these natural products, led to the deduction that two basic families existed:

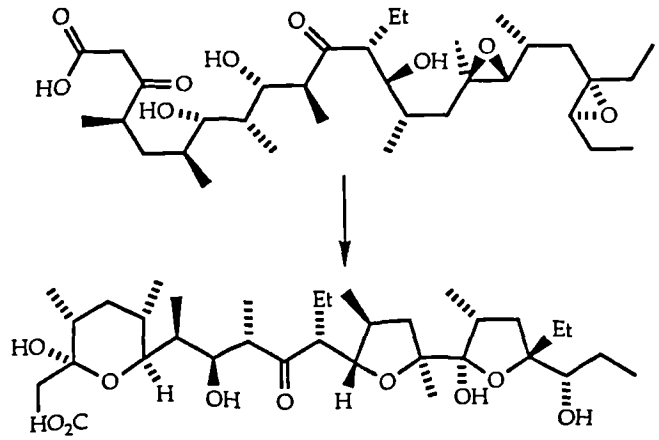
a) Those derived from initial acetate, propionate, propionate, acetate (APPA) condensation reactions, postulated to arise via a triepoxide precursor, eg monensin (**120**), scheme 3.1.6.



(120)

Scheme 3.1.6: Monensin (**120**) biosynthesis from a triepoxide initiated via an APPA condensation sequence.

b) Those derived from intial propionate (or butanoate), acetate, propionate (or butanoate), acetate (PAPA) condensation reactions, postulated to arise via a diepoxide precursor, eg lysocellin (**121**), scheme 3.1.7.

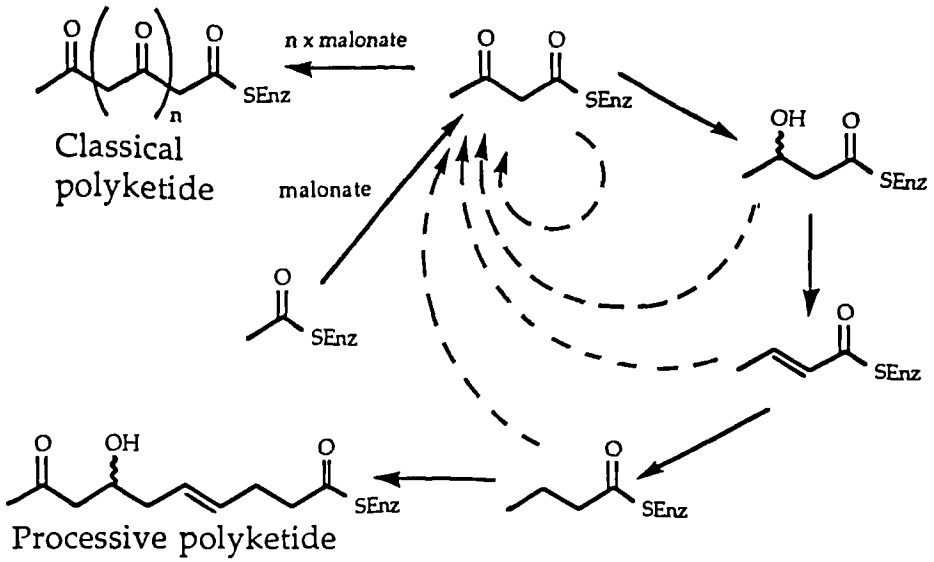


(**121**)

Scheme 3.1.7: Lysocellin (**121**) biosynthesis from a diepoxide initiated by a PAPA condensation sequence.

Further structural considerations of the macrolides and the polyether antibiotics, in particular the putative precursors to both, have shown a great deal of stereochemical homology between these two classes of metabolite. This has led to the suggestion that such natural products have a common evolutionary ancestor. Structural variation is postulated to have arisen as a result of specific environmental pressures upon the producing organisms.[13]

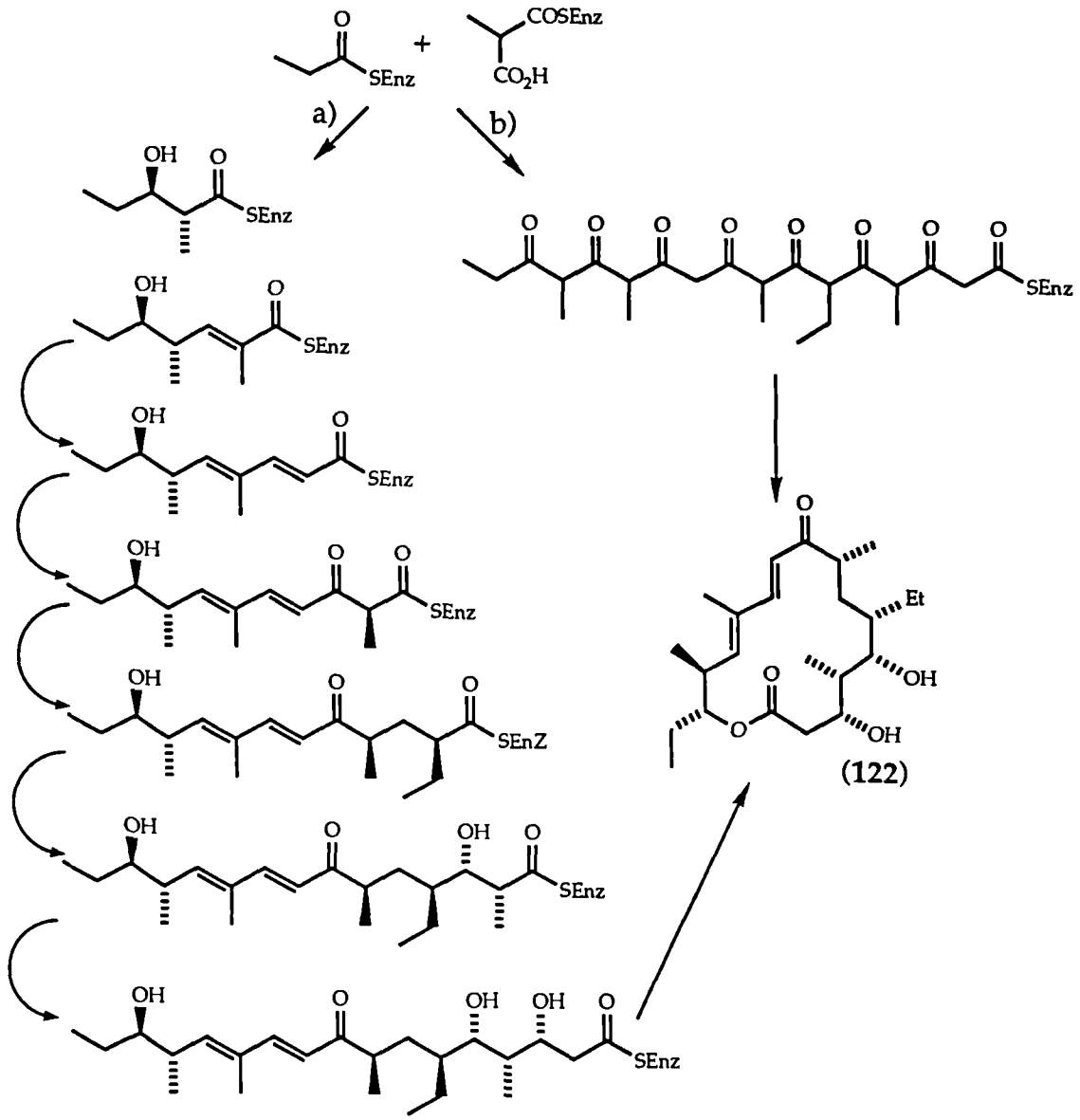
The epoxy precursors were the initial result of modifications after the polyketide chain elongation operations. The classical polyketide hypothesis for such epoxy precursors involves the formation of a poly-β-keto chain of the required length, followed by reductive modifications leading to the relevant intermediate. However, it was plausible that modifications could also occur contemporaneously with chain extension. In effect, such a processive pathway is directly analagous to fatty acid biosynthesis but with the proviso that stereochemical variation is possible, and each cycle need not be completed before chain elongation, as shown in scheme 3.1.8.



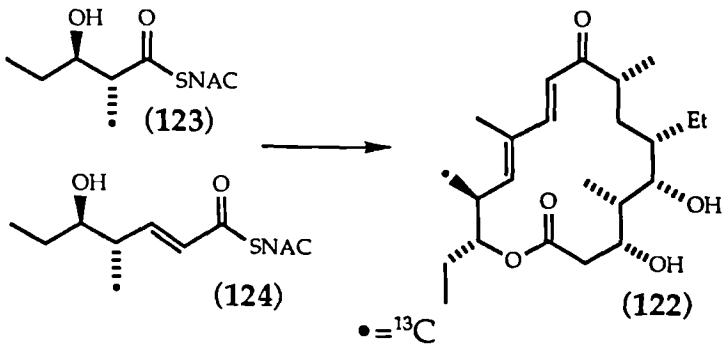
Scheme **3.1.8**: Alternative polyketide biosynthetic hypotheses.

These new ideas were first postulated by Hutchinson for the study of the biogenetic origin of the macrolide tylactone (**122**).[14] Scheme 3.1.9. shows the alternative biosynthetic pathways to this metabolite. Path (a) is that which would result were a processive mechanism in operation whereas path (b) is that predicted by classical polyketide ideas. Discrimination between the two pathways was reasoned to be experimentally possible via the specific incorporation of proposed intermediates along either pathway.

To this end the adminstration of [6-^{13}C]-(2R, 3R)-2-methyl-3-hydroxy pentanoate (**123**) to cultures of *Streptomyces fradiæ* was investigated.[14] Initial studies utilising the free acid or the sodium salt of this moiety led to the random incorporation of label, consistent with the operation of catabolic processes. The use of N-acetylcysteamine thioesters for the *in vitro* study of fatty acid biosynthesis was discussed in chapter one, and it was postulated that the use of such compounds would promote the *in vivo* study of polyketide biosynthesis. Thus the N-acetylcysteamine thioester (**123**) was administered to cultures and the isolated tylactone (**122**) analysed by ^{13}C nmr spectroscopy. This showed the specific incorporation of label into C-18, as predicted by the processive hypothesis, although concomitant catabolism had also taken place. Further evidence for this pathway operating in the biosynthesis of tylactone (**122**) was gained from the intact incorporation of [9-^{13}C]-(2E, 4R, 5R)-2,4-dimethyl-5-hydroxyhept-3-enoate (**124**) into the isolated metabolite.[14] These results are summarised in scheme 3.1.10.

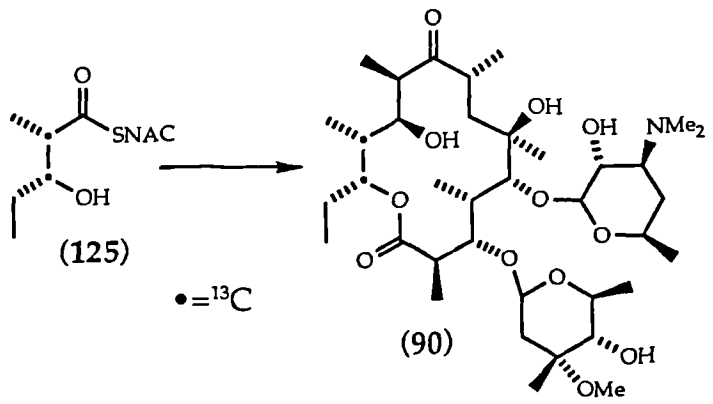


Scheme 3.1.9: Hypotheses for the biosynthesis of tylactone (122)



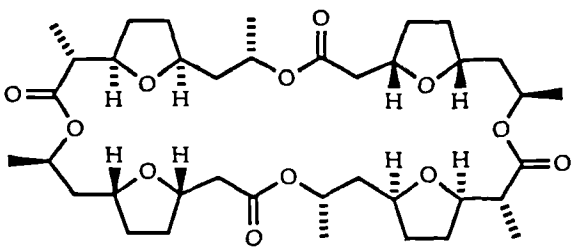
Scheme 3.1.10: Incorporation of chain intermediates into tylactone (122).

Investigations by Cane into the biosynthesis of erythromycin B (90) produced analagous results, thus presenting further evidence for the processive hypothesis. It was shown that [2,3-$^{13}C_2$]-(3S, 2R)-2-methyl-3-hydroxpentanaote (125) was incorporated intact into isolated erythromycin B (90) after adminstration to cultures of *Streptomyces erythreus*,[15] scheme 3.1.11. The use of the doubly labelled compound meant that intact incorporation was demonstrated by the presence of ^{13}C-^{13}C couplings in the ^{13}C nmr spectrum. Had catabolism taken place, such couplings would not have been observed.

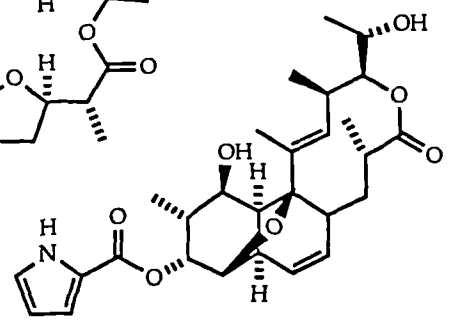


Scheme 3.1.11: Incorporation of a chain elongation intermediate into erythromycin B (90)

Evidence regarding the processive hypothesis of polyketide metabolism was further produced by the incorporation of putative triketide precursors into nonactin (126)[16] and nargenicin (127).[17]



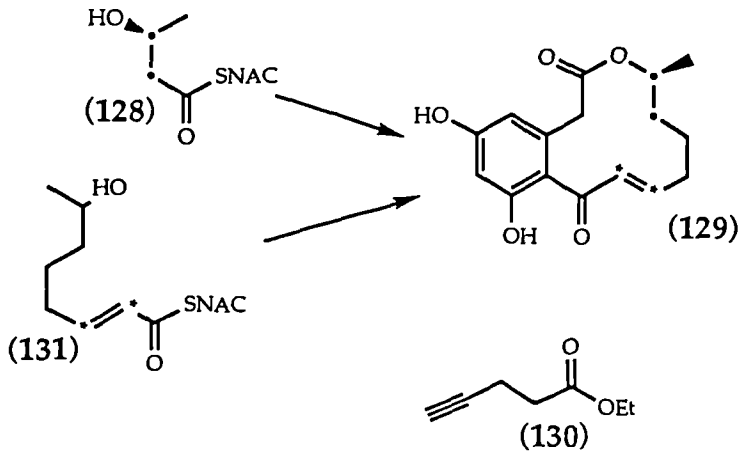
(126)



(127)

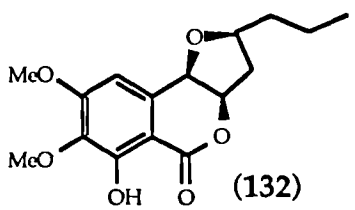
However, the use of the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-(3S)-3-hydroxybutanoate (128) to investigate the biogenesis of

dehydrocurvularin (129) (a metabolite from the fungus *Alternariæ cinerariæ*) led to complete catabolism upon administration.[18]This seemed initially anomalous with respect to the other reports, but further work involving the use of U.V. mutants unable to utilise fatty acids as a food source, the use of pent-4-ynoic acid (130) as a β-oxidation inhibitor and a high glucose replacement medium finally led to intact incorporation of (128) into dehydrocurvularin (129).[18] Under such conditions the intact incorporation of racemic [1,2-$^{13}C_2$]-(2E)-7-hydroxyoct-2-enoate (131) was also observed, albeit with concomitant catabolism,[18] scheme 3.1.12. This was the first demonstration that a processive process operates in a fungus, as well as streptomycetes.



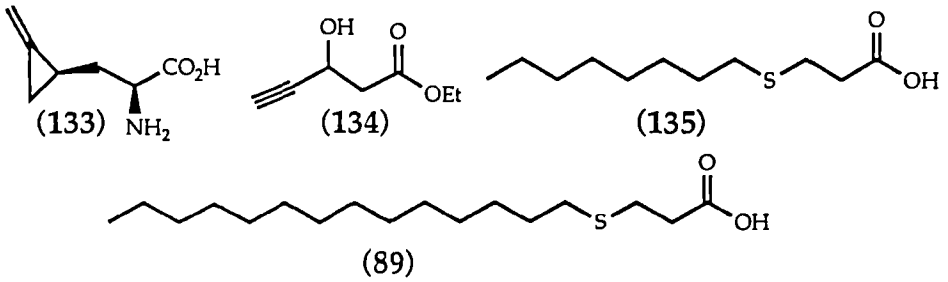
Scheme 3.1.12: Incorporation of chain elongation intermediates into dehydrocurvularin (129),

The competition between catabolism and metabolism of administered compounds has already been described for the case of 9-hydroxynonanoic acid (45) in chapter two of this work. Other reports have similarly been made with respect to studies into the biosynthesis of monensin (120)[19] and monocerin (132).[20]



Further work on the biogenesis of dehydrocurvularin (129) demonstrated that a variety of β-oxidation inhibitors promoted the

increased incorporation of the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-(2E, 7S)-7-hydroxyoct-2-enoate (**131**) into the metabolite.[21] Amongst the most efficient were hypoglycin (**133**), ethyl-3-hydroxypent-4-ynoate (**134**) and octylthiopropionic acid (**135**), with the most efficient one being found to be tetradecylthiopropionic acid (**89**).



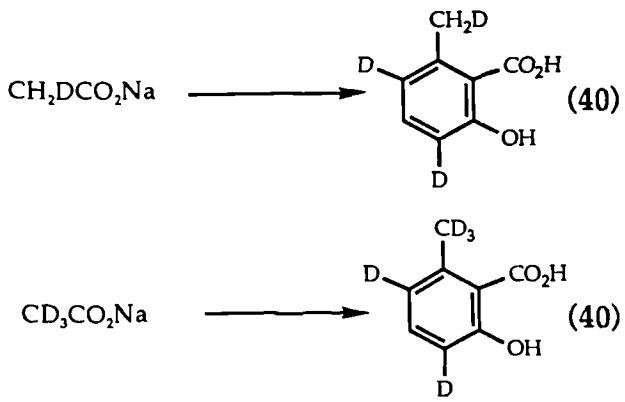
(133) (134) (135)

(89)

3.1.4: Stereochemistry of polyketide biosynthesis.

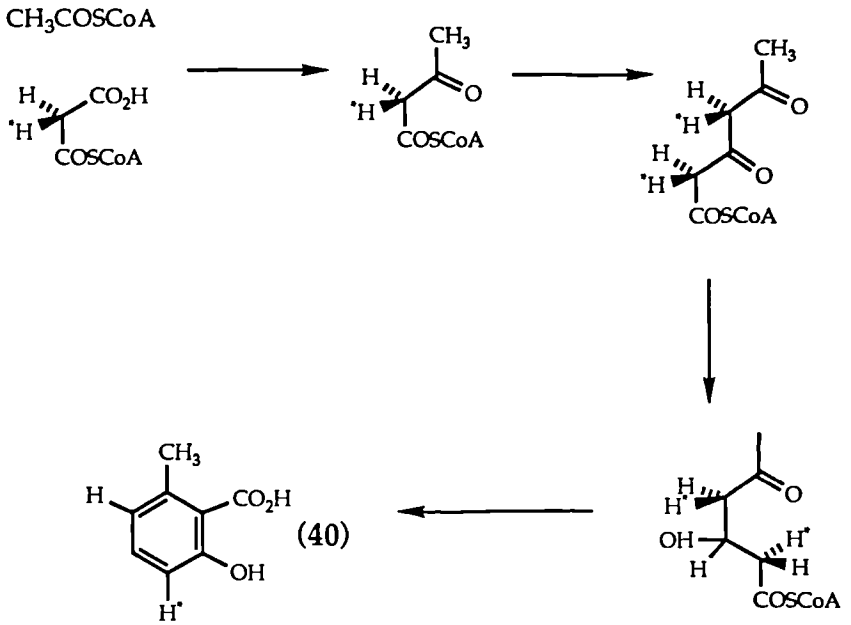
That variations in the stereochemical course of polyketide biogenetic processes between species are allowed can be inferred from the range of metabolites produced by this pathway.

Early studies on the stereochemistry of polyketide biosynthetic processes involved feeding mono- and tri-deuterated acetates to cultures of *Penicillium patulum*, a producer of 6-methylsalicylic acid (**40**).[22] It was found that the relative retention of labels at both C-3 and C-5 was the same in either case (scheme 3.1.13), thus indicating that the abstraction of hydrogen at these positions proceeded with no primary kinetic isotope effect. It was deduced, therefore, that the aromatisation step was under enzymatic control and therefore a stereospecific process.



Scheme 3.1.13: Incorporation of deuterated acetates into 6-methylsalicylic acid

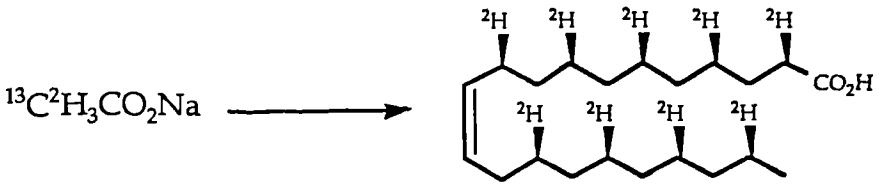
Further investigations on the stereochemical pathway to 6-methylsalicylic acid (40) were possible using purified polyketide synthases from *Penicillium* species.[23] The incubation of chiral malonic acids with this, in the presence of acetyl CoA (9) and succinyl CoA transferase (to catalyse the formation of malonyl CoA), was reported by Jordan.[24] The comparison of the experimentally derived mass spectrum of the isolated 6-methylsalicylic acid was compared to those predicted statistically. This showed C-3 and C-5 of 6-methylsalicylic acid (40) were derived from hydrogens of opposite absolute configuration in malonate.



Scheme 3.1.14: Stereochemical pathway to 6-methylsalicylic acid (40)

Experimental determination of the origin of C-3 (and thus by implication C-5) was made by the substitution of acetoacetyl CoA (27) for acetyl CoA (9) as the starter unit, as under such conditions only C-3 would be labelled. Mass spectral analysis showed that C-3 of the isolated metabolite originated from H_{re} of malonate, and therefore C-5 was from H_{si}. A stereochemical pathway to 6-methylsalicylic acid (40) was postulated, as shown in scheme 3.1.14.

Experimental evidence regarding the control of stereochemistry in the chain elongation process was achieved by Vederas et al, by comparative studies of polyketide and fatty acid processes in the species *Alternia cinerariæ*,[25] *Penicillim turbatum*[25] and *Cladosoprium cladosporioides*.[26] In particular the enoyl reductase step in both pathways was investigated.

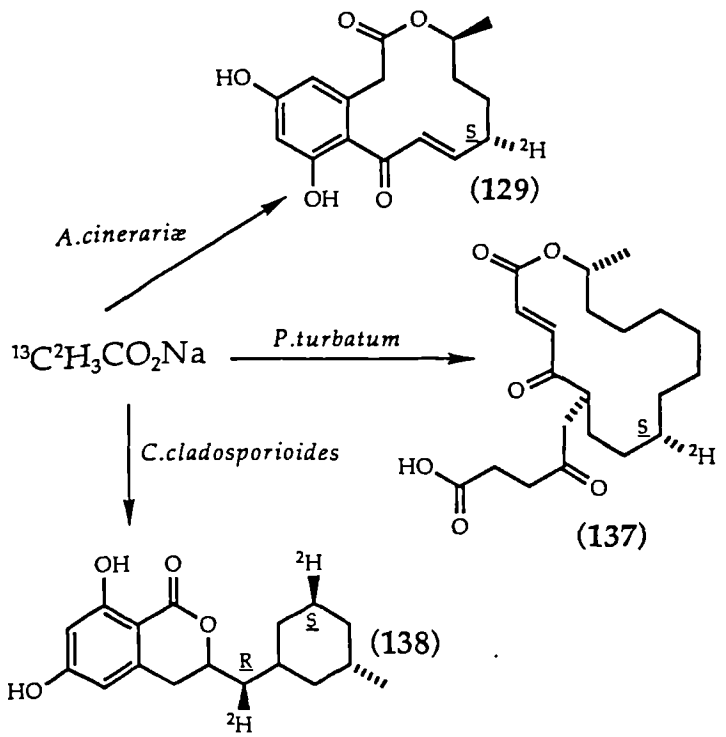


(136)

Scheme **3.1.15**: Incorporation of deuterium from labelled acetate into the pro-R positions during fatty acid biosynthesis

In all three species it was found that after adminstration of [2-^{13}C,$^2H_3$] acetate the deuterium label occupied the pro-R positions along the carbon chain of oleic acid (**136**),[25,26] scheme 3.1.15.

Similar experiments were carried out on the polyketides isolated from these fungii. Both C-7 of dehydrocurvularin (**129**, from *A. cinerariæ*) and C-8 of A26771B (**137**, from *P. turbatum*) were shown to have deuterium in the pro-S position.[25] For cladosporin (**138**, from C. *cladosporioides*), the deuterium was found to occupy the pro-S position at C-11, but the pro-R position at C-9.[26] These results are summarised in scheme 3.1.16.



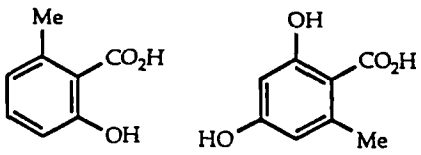
Scheme **3.1.16** : Differential incorporation of deuterium in the course of polyketide biosynthesis.

These studies demonstrated not only the variation of the stereochemical course of polyketide metabolism between species, but that within one species fatty acid and polyketide biosynthetic modes may follow different stereochemical pathways. It was also demonstrated that the polyketide pathway towards one metabolite within a species exhibited varying stereochemical operations.

3.1.5: The enzymology and genetics of polyketide biosynthesis.

There is growing evidence that the polyketide and fatty acid biosynthetic pathways are, at least partially, controlled by the acyl carrier proteins (ACP).[27] The three-dimensional structure of an *Escherischia coli* ACP has been elucidated by nmr methods,[28] and has been shown to include a hydrophobic cleft suggested to be a possible site for the binding of acyl chains. A heat stable factor known to be a pre-requisite for the biosynthesis of fatty acids in *Saccharopolyspora erythrea* has been isolated and shown to be a discrete ACP.[29] This suggests that the FAS of *S. erythrea* is a dissociable complex, similar to that of *E. coli.*

By analogy to fatty acid biosynthesis, the enzymes that catalyse polyketide biosynthetic processes are termed polyketide synthases (PKS). To date only two have been isolated, namely 6-methylsalicylic acid (**40**) synthase from *Penicillium* species[23] and orsellinic acid (**38**) synthase from *Penicillium cyclopium.*[30] These were both shown to be polyfunctional proteins.

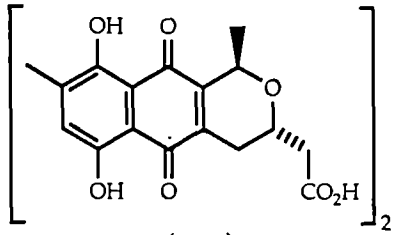


(**140**) (**38**)

Studies on the genetics underlying the polyketide biosynthetic process leading to actinorhodin (**139**), produced by *Streptomyces coelicolor,* indicated the presence of discrete genes coding for the different biosynthetic operations. This implies that individual non-covalently linked enzymes catalyse the biosynthesis of this metabolite.[31]

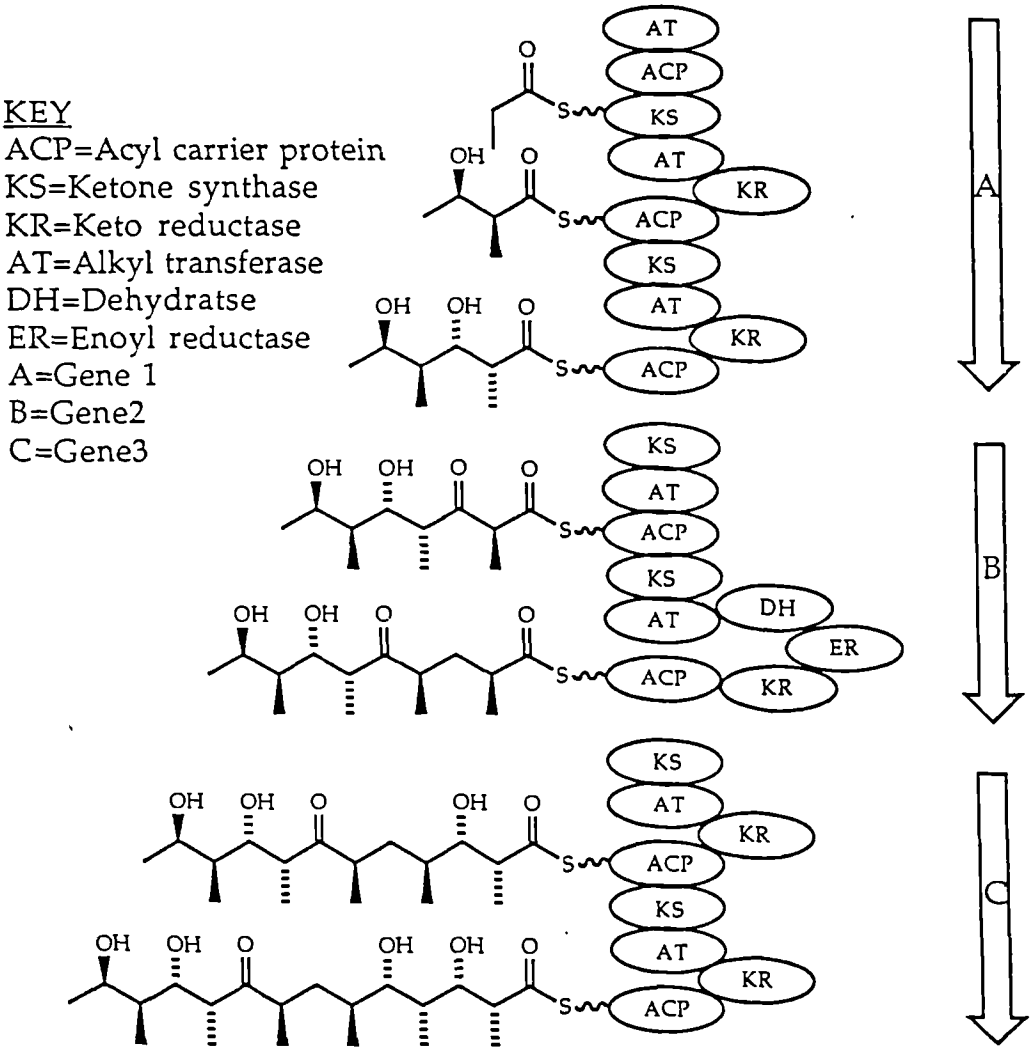
The study of the genetics of *S. erythrea,* the erythromycin B (**90**) producing organism, has led to further information regarding the

enzymatic processes involved.[32] From this work it is clear that the biosynthesis of erythromycin B (90) is an elaborately controlled process



(139)

Study of the DNA around the resistance gene led to the ultimate discovery of three PKS genes, each of which code for large multi-functional proteins capable of carrying out two chain extension cycles.



Scheme 3.1.17: The extension of the polyketide chain in erythromycin biosynthesis, from the expression of three genes, and the enzymes which carry out these processes.

The implication from these studies is that for each individual step on the biosynthetic pathway to erythromycin B (80) there exists an individual catalytic domain. These domains appear to be linked into six groups, and further grouped into 'cassettes' that lead to the production of 6-deoxyerythronolide B (91) as the first 'enzyme free' intermediate, as shown in scheme 3.1.17.

Thus there appears to be 2 classes of polyketide synthase enzyme:

a)Type I polyketide synthases exist as single multifunctional proteins eg 6-methylsalicylic acid (40) synthase,

b)Type II: polyketide synthases consist of a series of individual enzymes eg actinorhodin (139) synthase

The 'cassette compilation' of discrete enzymes, as in the case for erythromycin, appears to bve intermediate between these two.

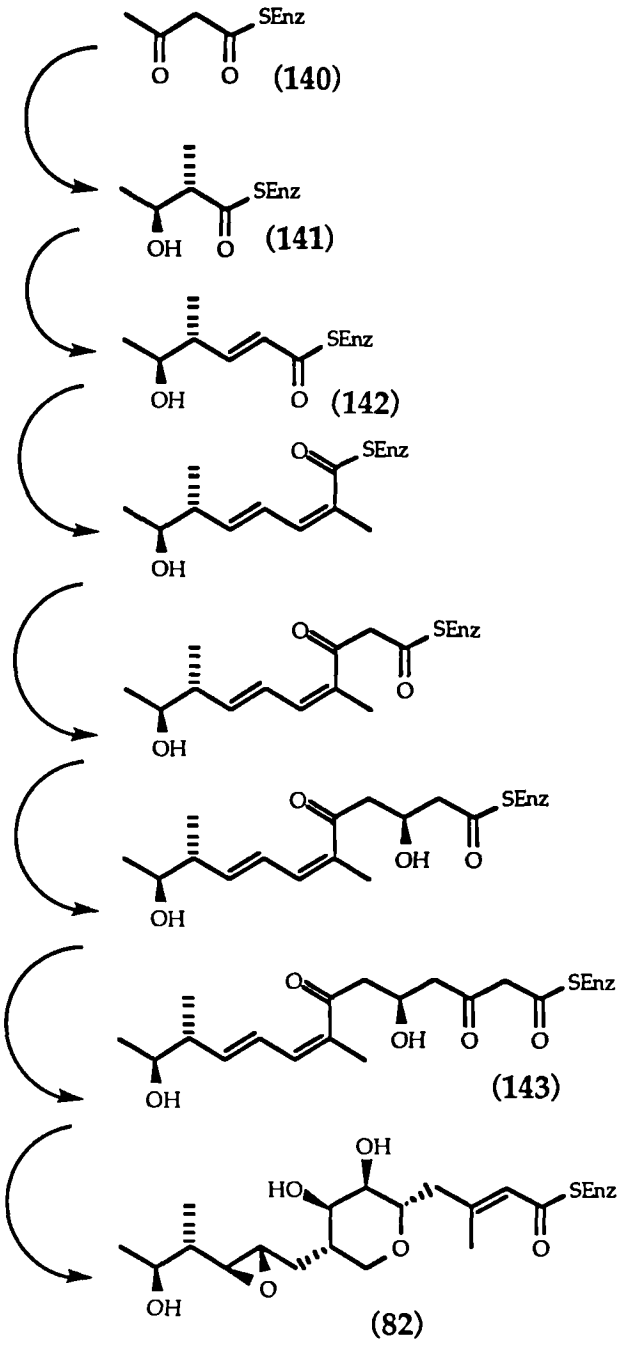
3.1.6: Application of the processive hypothesis to monic acid (84)

Using this hypothesis a step-wise biosynthetic pathway can be postulated for the biosynthesis of monic acid (84) as shown in scheme 3.1.18. The initial acetate/malonate condensation produces enzyme bound acetoacetate (140), which undergoes α-methylation and then reduction of the β-keto function to give a (2S, 3S)-3-hydroxy-2-methylbutanoyl enzyme bound moiety (141). A further condensation of malonate onto this causes chain extension to an enzyme bound hexanoyl moiety. β-reduction and subsequent dehydration leads to the (2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate enzyme bound intermediate (142). Further chain extension via malonate condensation reactions and appropriate reductive modifications between each condensation step ultimately leads to the putative heptaketide precursor (143) to monic acid (84)

In order to determine the validity of this scheme, the first three intermediates on the pathway, acetoacetate (140), (2S, 3S)-3-hydroxy-2-methylbutanaoate (141) and (2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (142) were chosen for incorportion studies.

For this to be possible efficient syntheses of isotopically labelled forms of these compounds as their N-acetylcysteamine thioesters were required.

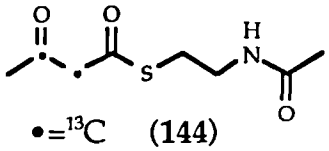
The choice of position of label was important, to ensure experiments could distinguish between catabolism and intact incorporation.



Scheme 3.1.18: Processive biosynthesis of monic acid (84)

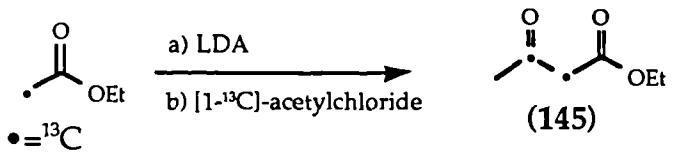
3.1.7: Synthesis of the N-acetylcysteamine thioester of acetoacetate (144)

It was decided that double isotopic labelling of this moiety would produce the desired experimental sensitivity. As was discussed in the case for the N-acetylcysteamine thioester of 9-hydroxynonanoic acid (**106**), labelling of a bond equivalent to that formed by an acetate/malonate condensation process leads to the highest sensitivity. This meant that the required target molecule was the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-acetoacetate (**144**).



•=^{13}C (**144**)

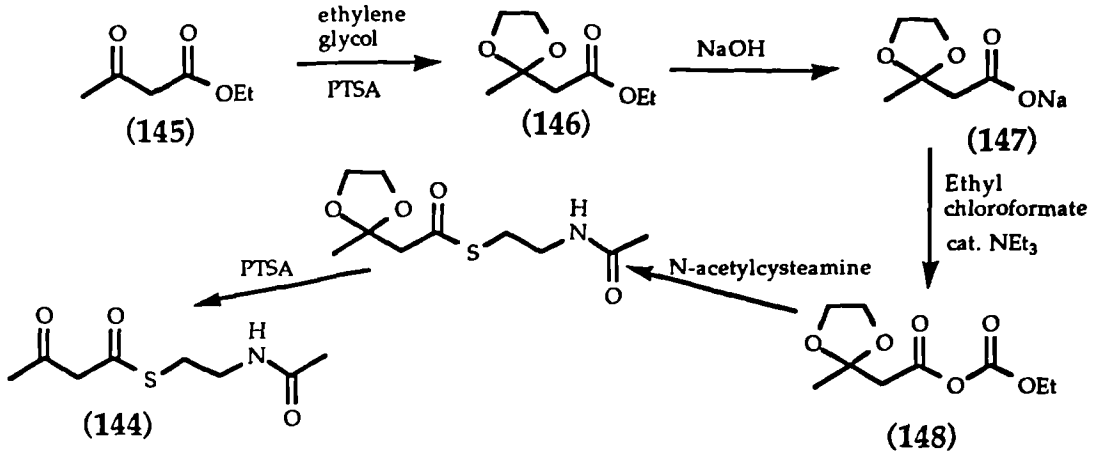
Introduction of labels was possible by the use of ethyl [2-^{13}C]-acetate and [1-^{13}C]-acetyl chloride, as shown in scheme 3.1.19, to produce ethyl [2,3-$^{13}C_2$]-acetoacetate (**145**) in 71% yield. This left conversion of the ethyl ester into the relevant thioester as the target of synthetic studies.



Scheme 3.1.19: Synthesis of ethyl [2,3-$^{13}C_2$]-acetoacetate (**145**).

Previous work by Martin[33] had led to the development of the synthesis outlined in scheme 3.1.20. This involved initial protection of the ketone function as its ethylene ketal derivative (**146**) prior to saponification to the sodium salt (**147**). Activation of the carbonyl group via mixed anhydride (**148**) formation preceeded introduction of the N-acetylcysteamine (**98**) entity. Deprotection led to the final product (**144**).

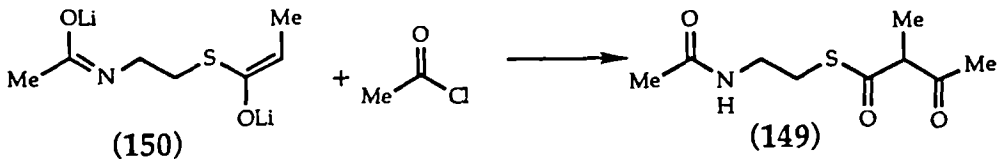
This scheme was reinvestigated. It was found that under the described conditions (2 equivalents ethyl chloroformate, catalytic triethylamine) the formation of the mixed anhydride (**148**) gave erratic yields. Replacement of triethylamine by DMAP led to reliable yields of 61%. Although the introduction of N-acetylcysteamine (**98**) by way of the mixed anhydride (**148**) proceeded well in high yield (80%), the deprotection reaction involved the use of catalytic PTSA, and the thioester function was sensitive to this reagent. Yields of 60% were never improved upon.



Scheme 3.1.20: Martin's synthesis of the N-acetylcysteamine thioester of acetoacetate (142)

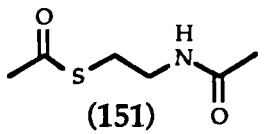
Overall, although this scheme led to production of the required product it is rather lengthy for such an intrinsically simple conversion, and led to a high waste of material. This is an important factor when expensive labels are to be used. It was therefore decided to investigate alternative strategies.

For the study of monensin (120) biosynthesis Cane and Block synthesised the N-acetylcysteamine thioester of 2-methylacetoacetate (149) from the di-lithium enolate of N-acetyl-S-propylcysteamine (150) and acetyl chloride,[34] scheme 3.1.21. Although the recovery of product was low (33%) the required compound was formed in the one step making this a particularly attractive approach.



Scheme 3.1.21: The Cane synthesis of N-acetylcysteamine thioesters.

It was hoped that by use of the di-lithium enolate of N,S-diacetylcysteamine (151), the N-acetylcysteamine thioester of acetoacetate (144) could be synthesised in a similar fashion. However, neither the use of LDA nor LHMDS for generation of the dienolate led to formation of the required compound, with the N,S-diacetylcysteamine (151) being returned.



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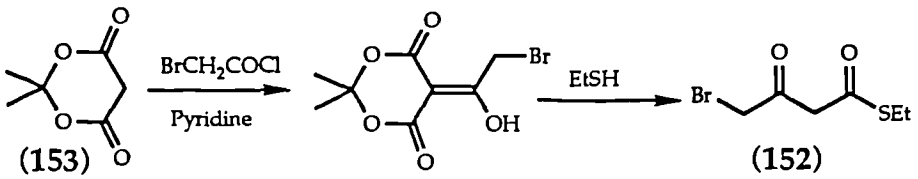
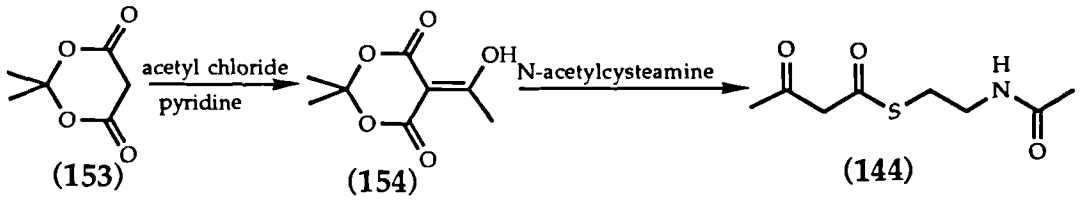
The synthesis of the ethyl thioester of 4-bromoacetoacetate (152) from Meldrum's acid (153) was reported by Ley in 1987,[35] as shown in scheme 3.1.22.



(153) (152)

Scheme 3.1.22: Ley's synthesis of ethyl thioesters.

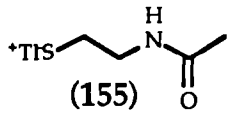
It was reasoned that the use of acetyl chloride to form the acetyl Meldrum's acid derivative (154), followed by reaction with N-acetyl cysteamine (98) would lead to formation of the desired product (144).[36] Being as Meldrum's acid (153) is itself made from malonate,[37] the use of [2-^{13}C] malonate and [1-^{13}C]-acetyl chloride would lead to introduction of the required labels, scheme 3.1.23.



(153) (154) (144)

Scheme 3.1.23: Proposed synthesis of the N-acetylcysteamine thioester of acetoacetate (144) from Meldrum's acid (153)

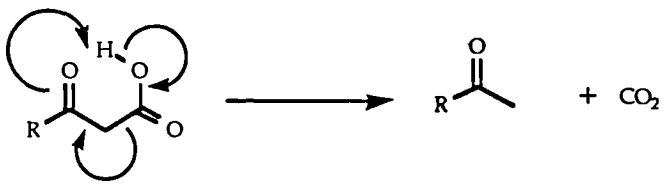
However, although the required acyl Meldrum's acid derivative (154) was easily synthesised the reaction with N-acetylcysteamine (98) led only to return of the reactants, even with the use of five equivalents of thiol in refluxing chloroform. Since thiols are 'soft' nucleophiles and the carboxylates of the Meldrum's acid is a 'hard' nucleophilic site, the use of a 'hard' sulphur was investigated, the thallium(I) thiolate of N-acetylcysteamine (155)[38]. However this also gave no production of the desired compound.



(155)

It is worth noting that since the investigation of this scheme was completed a report of the synthesis of the N-acetylcysteamine thioester of acetoacetate (**144**) by way of Meldrum's acid (**153**) has been made.[39] However no experimental details or yields were supplied.

At this point it was decided that re-investigation of the conversion of the ethyl ester (**145**) to the N-acetylcysteamine thioester (**144**) might be beneficial. A number of methods exist in the literature for the conversion of acids to thiols.[40] However this would require the use of a β-ketoacid for the formation of the N-acetylcysteamine thioester of acetoacetate (**144**), entities which are known to be labile owing to facile de-carboxylation with the ketone acting as an electron sink,[41] scheme 3.1.24. Only two syntheses of acetoacetic acid (**156**) could be found in the literature. One of these was of a concentrated ethereal solution,[42] whilst the other was of a crystalline form but yields of only 10% were reported.[43]

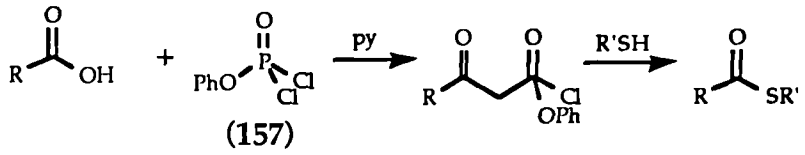


Scheme 3.1.24: The decarboxylation of β-keto acids

It was found that the careful hydrolysis of distilled ethyl acetoacetate (**145**) using sodium hydroxide at 5°C followed by slow acidification at 0°C and multiple extractions with cold ethyl acetate produced acetoacetic acid (**156**) in 93% yield. At room temperature this was a colourless liquid that soon decomposed. However upon placement in a freezer the acid solidified and was stable long periods. ^{1}H nmr spectral analysis of this revealed it to be 85-95% pure with the enol tautomer accounting for ca 15% of this.

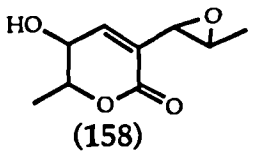
Initial investigations into the direct conversion of this acid (**156**) into the N-acetylcysteamine thioester of acetoacetate (**144**) were made by the use of the phenyl dichlorophosphate (**157**) method.[44] In conjunction with pyridine, this leads to the *in situ* activation of the acid function via phosphoanhydride formation such that attack by the thiol is enhanced,

scheme 3.1.25. However under the conditions described the desired product was not formed. Reduction of the reaction temperature to -50°C, with overnight warming to room temperature, led to no product formation.

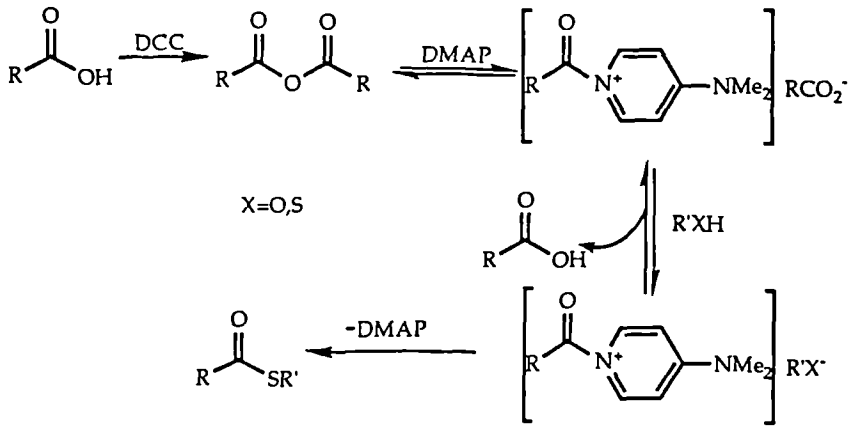


Scheme 3.1.25: Course of the thioesterification of acids mediated by phenyl dichlorophosphate **(157)**

The synthesis of thioesters for the study of aspyrone **(158)**[45] and nonactin **(127)**[19] biosynthesis was achieved by the use of DCC coupling reactions. This reagent is often used for the synthesis of both oxy- and thioesters in conjunction with DMAP as a catalyst.[46,47] The postulated mechanism for this reaction[47,48] is shown in scheme 3.1.26.

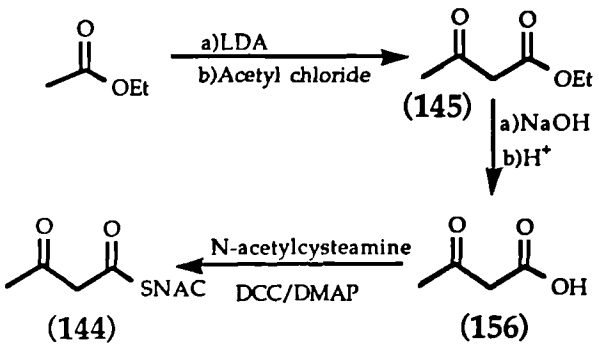


The use of this method to synthesise the N-acetylcysteamine thioester of acetoacetate (144) was found to be unproductive. Replacement of DMAP with pyridine,[49] however, and the use of a -25°C starting temperature gave the required product in 71% yield.



Scheme 3.1.26: Course of DCC/DMAP mediated esterifications

The complete synthesis of the N-acetylcysteamine thioester (144) is displayed in scheme 3.1.27, and the ^1H nmr spectra of the labelled and unlabelled compounds are shown in figure 3.1.1.



Scheme 3.1.27: Synthesis of N-acetylcysteamine thioester of acetoacetate (144)

It was found that purification of the N-acetylcysteamine thioester of acetoacetate (144) on silica led to substantial hydrolysis of the thioester bond. This was reduced by pre-treating the silica with tri-ethylamine, and the use of preparative TLC as opposed to flash column chromatography also reduced the problem.

31.8: Synthesis of the N-acetylcysteamine thioester of (2S,3S)-3-hydroxy-2-methylbutanoate (159)

The branching methyl group of (159) is postulated to correspond to C-17 of monic acid (84) by the processive hypothesis (scheme 3.1.18). This carbon is known to be derived but from methionine. Therefore should any isotopically labelled compound undergo β-oxidation after administration to *Pseudomonas fluorescens*, the label would not be expected to appear at C-17 of the isolated metabolite as such processes lead only to acetate production. Hence if label is introduced into the branching group of (2S, 3S)-3-hydroxy-2-methylbutanoate (159), detection of any label at C-17 would imply intact incorporation of this compound into the metabolite. By this reasoning the desired target molecule therefore became the N-acetylcysteamine thioester of [5-$^2H_3$]-(2S, 3S)-3-hydroxy-2-methylbutanoate (159), with label introduced via [$^2H_3$]-iodomethane.

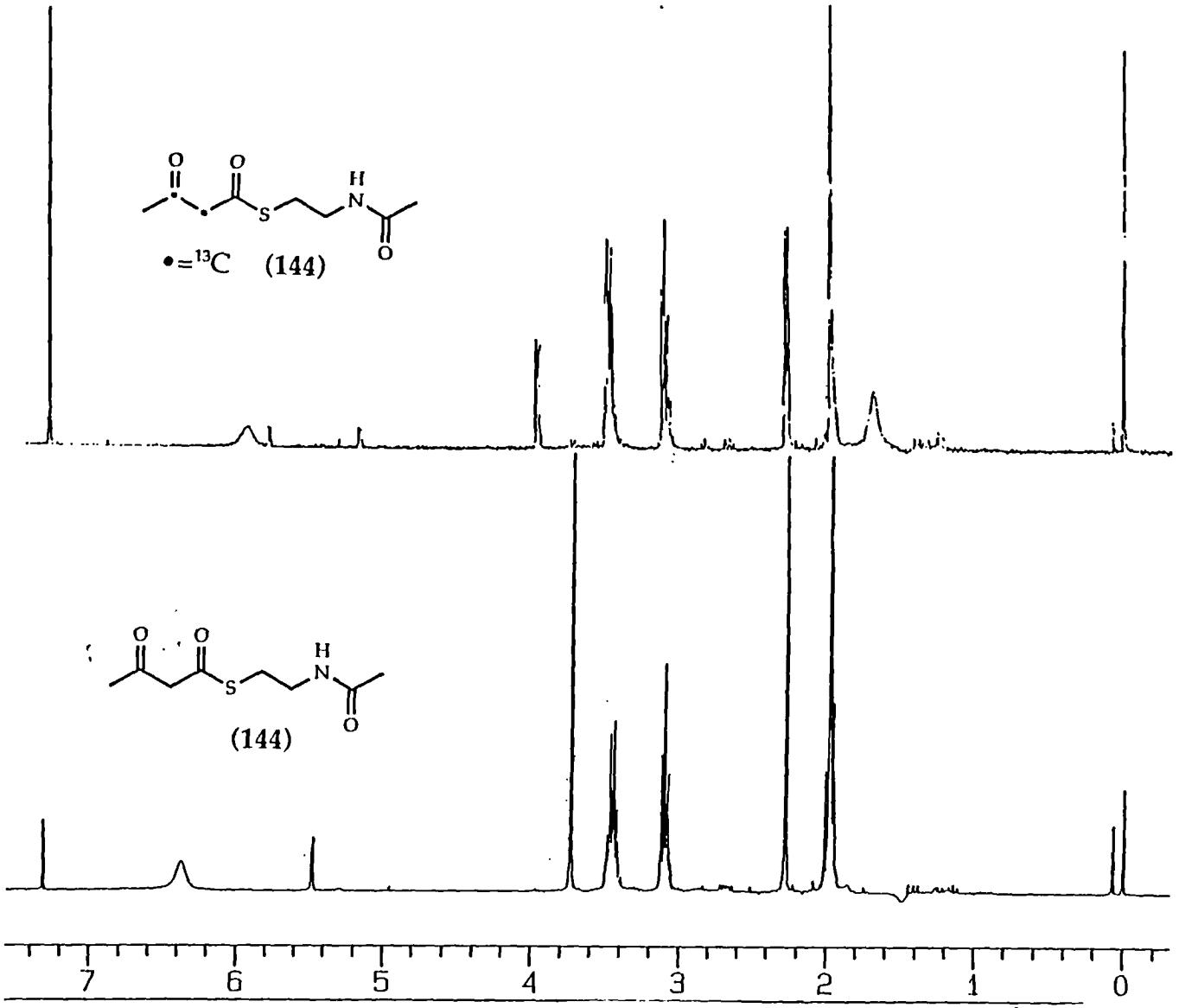
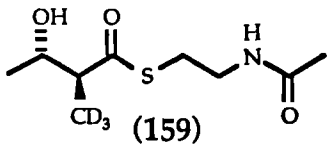
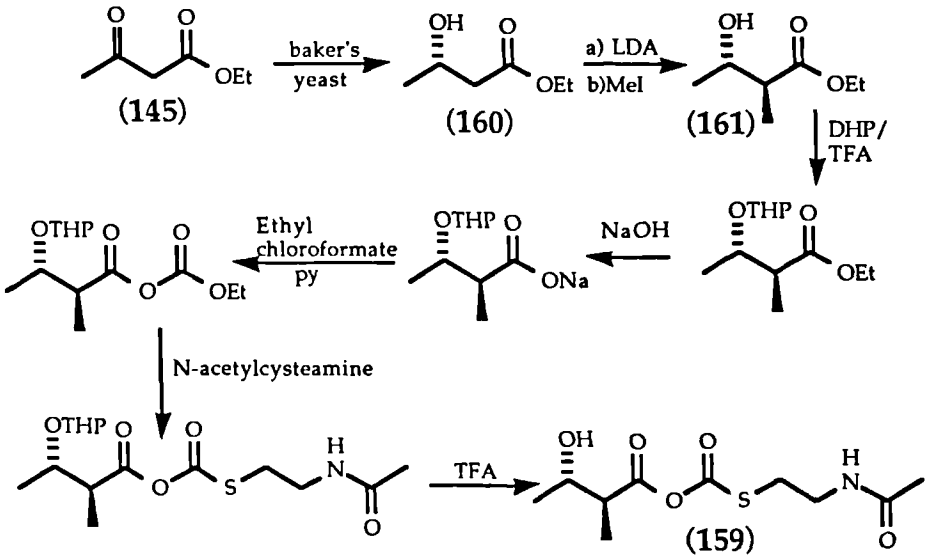


Figure 3.1.1: ^{1}H nmr spectra of unlabelled and labelled N-acetylcysteamine thioesters of acetoacetate **(144)**.



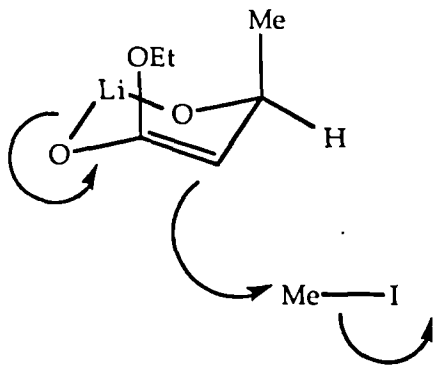
(159)

Again, earlier work by Martin[33] had led to the development of a synthesis of this compound, as shown in scheme 3.1.28. The required stereochemistry was introduced by means of a baker's yeast reduction of ethyl acetoacetate (145) to produce ethyl (3R)-3-hydroxybutanoate[50] (160) in 56% yield, followed by asymmetric methylation.[51]



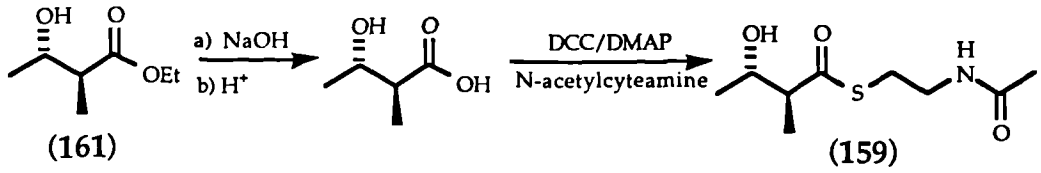
Scheme 3.1.28: Martin's synthesis of the N-acetylcysteamine thioester of (2S, 3S)-3-hydroxy-2-methylbutanoate (159)

As shown in scheme 3.1.29, the presence of the methyl group causes facial bias in the electrophilic attack of the methyl group.



Scheme 3.1.29: Asymmetric induction in the methylation of (3R)-3-hydroxybutanoate (158).

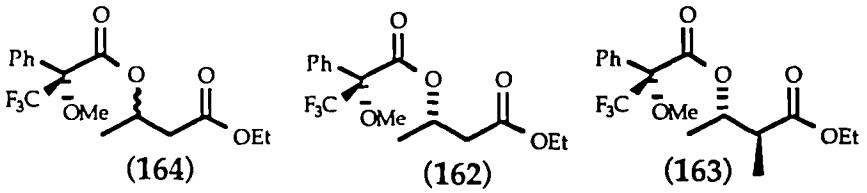
At this point Martin protected the hydroxy function prior to taking a series of operations directed to introduction of the N-acetylcysteamine (98) moiety. From the work already undertaken on the synthesis of the N-acetylcysteamine thioester of acetoacetate (144) it was decided to investigate a more direct route, scheme 3.1.30. Thus ethyl (2S,3S)-3-hydroxy-2-methyl butanaote (161) was saponified to the free acid in 86% yield which was then thioesterified via the DCC/DMAP method[46] to give the required thioester (159) in 78% yield. The ^{1}H and ^{2}H nmr spectra of labelled and unlabelled material are shown in figure 3.1.2



(161) (159)

Scheme 3.1.30: Synthesis of the N-acetylcysteamine thioester of (2S, 3S)-3-hydroxy-2-methylbutanoateb (159)

The enantiomeric purity of the synthesis was verified by GLC of the Mosher's esters[52] of ethyl (3R)-3-hydroxybutanoate (162) and ethyl (3R, 3S)-3-hydroxy-2-methylbutanoate (163).



(164) (162) (163)

It was found that the baker's yeast reduction gave an ee of 89-93%, and that methylation yielded (161) in 82-85% de. The saponification process was found to induce no epimerisation. These results are in good agreement with the literature values.[50,51] The ^{1}H nmr spectra of these (figure 3.1.3) show the presence of predominantly one stereoisomer. The Mosher's ester of ethyl (rac)-3-hydroxybutanoate (164) is also shown for comparison.

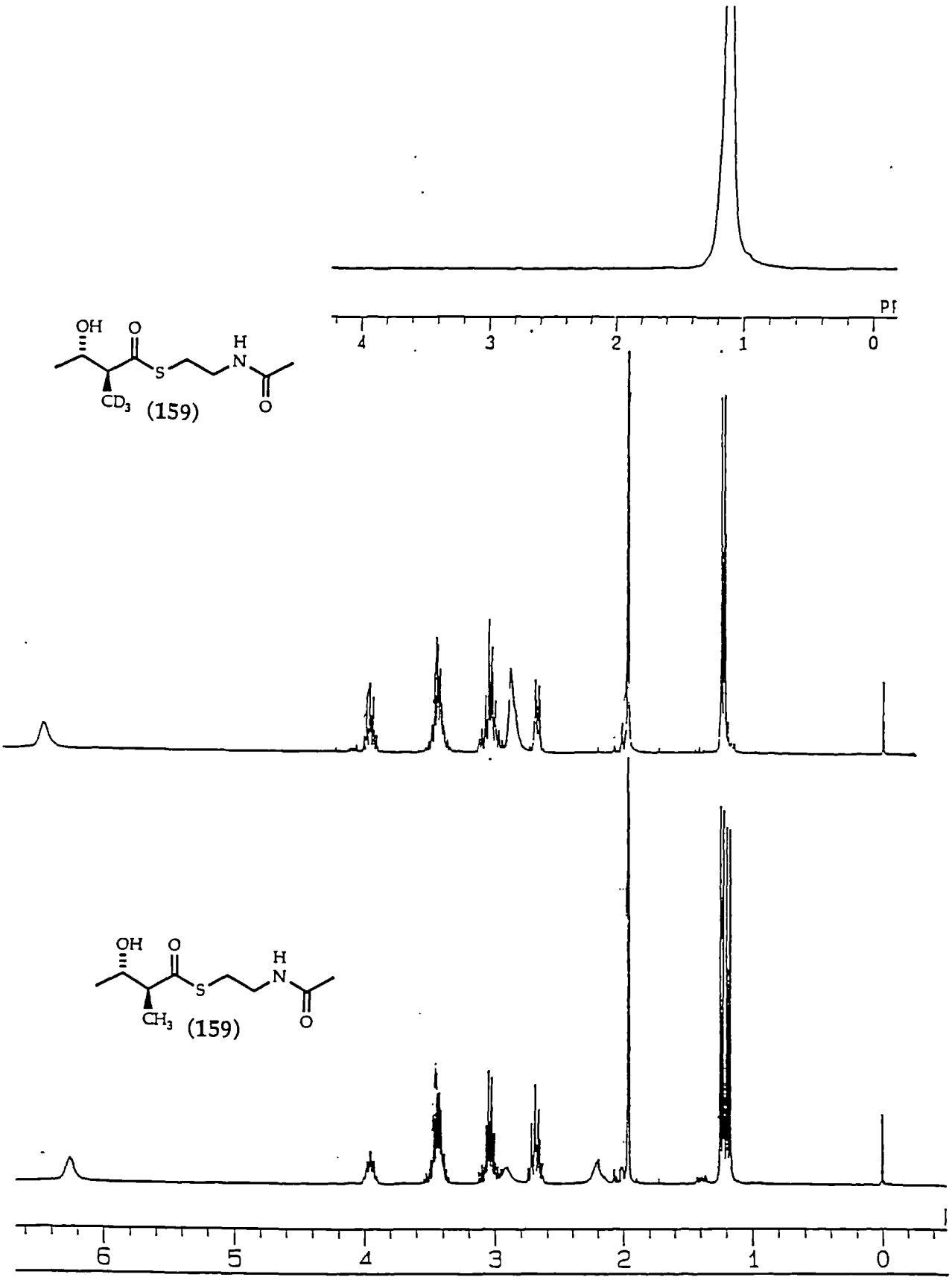


Figure 3.1.2: ^{1}H and ^{2}H nmr spectra of unlabelled and labelled N-acetylcysteamine thioester of (2S, 3S)-3-hydroxy-2-methylbutanoate (159).

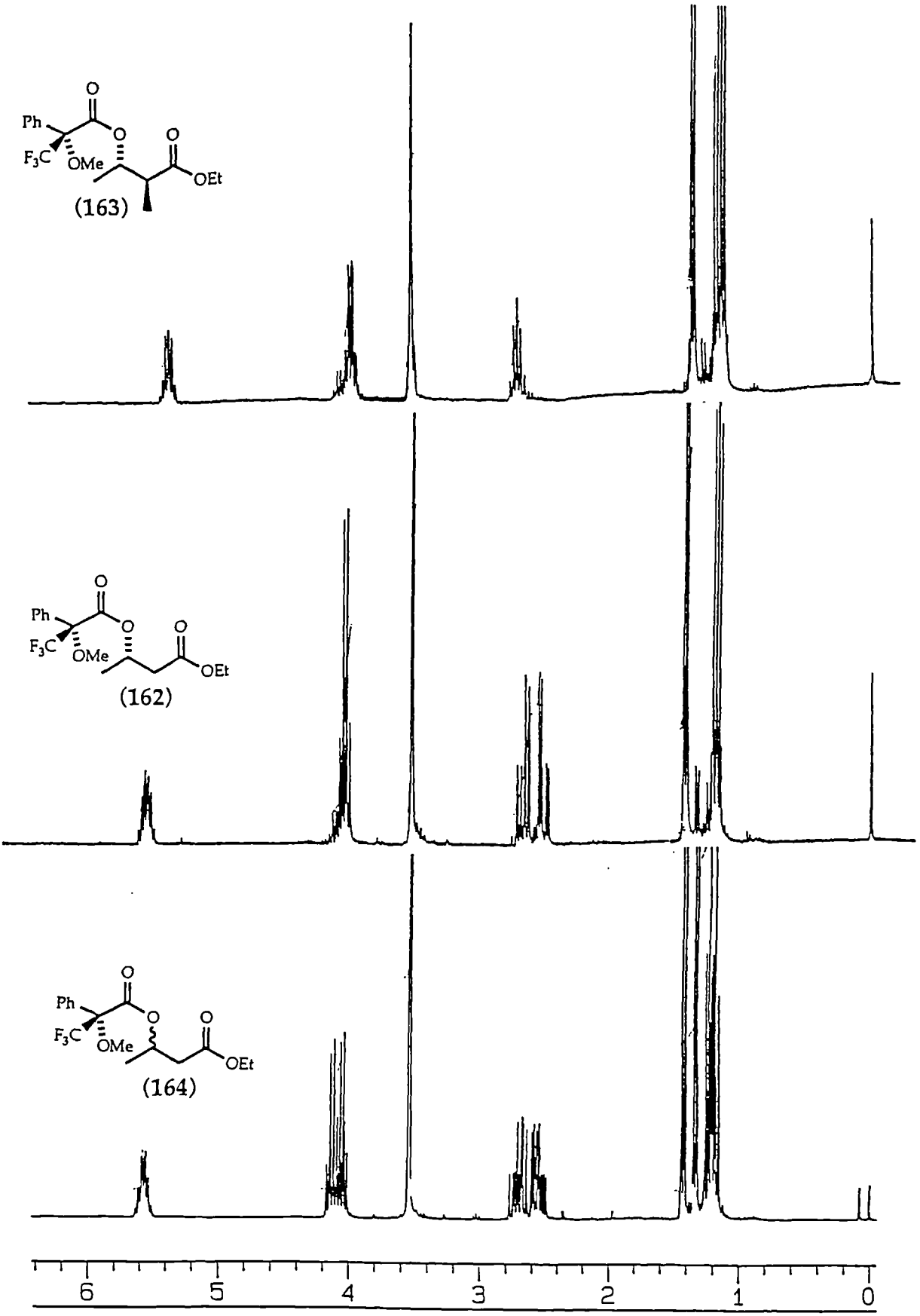
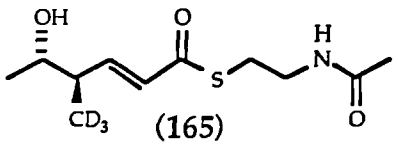


Figure 3.1.3: ^{1}H nmr spectra of Mosher's esters (162), (163) and (164).

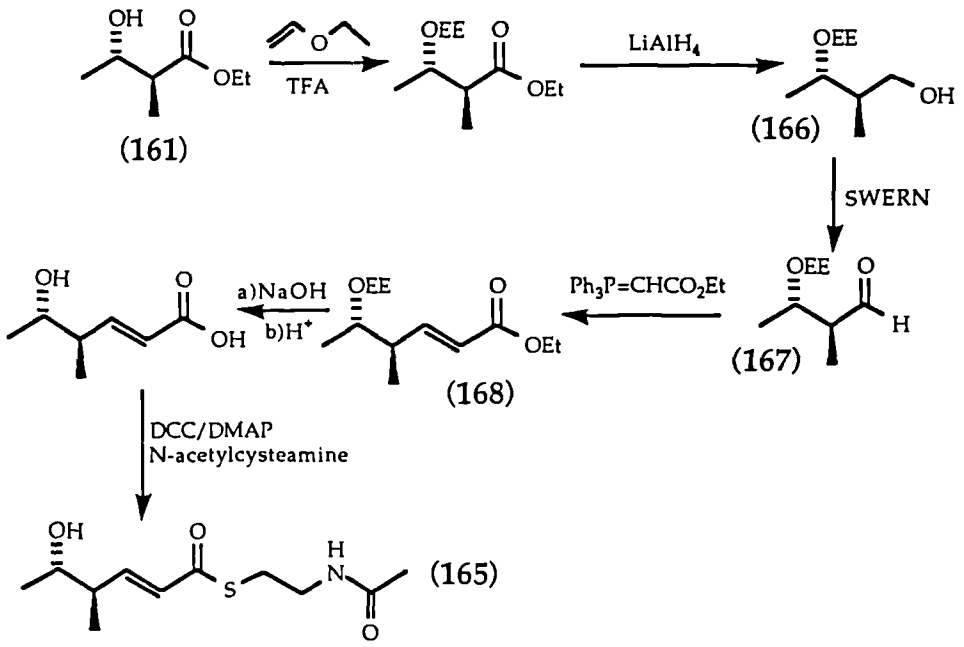
3.1.9: Synthesis of the N-acetylcysteamine thioester of (2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (165)

By the same reasoning to that described in the previous synthesis, the target molecule for this synthesis was the N-acetylcysteamine thioester of [7-$^2H_3$]-(2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (**165**). The asymmetry within the molecule was introduced as before such that the starting point for this synthesis was ethyl (2S, 3S)-3-hydroxy-2-methylbutanoate (**161**).



(165)

A linear approach to this was first investigated, as shown in scheme 3.1.31. Thus ethoxyethoxy protection of the hydroxy entity within (**161**) preceeded reduction with Lithium aluminium hydride to produce (2S,3S)-3-(1-ethoxy)ethoxy-2-methylbutanoate (**166**) in 83% overall yield.

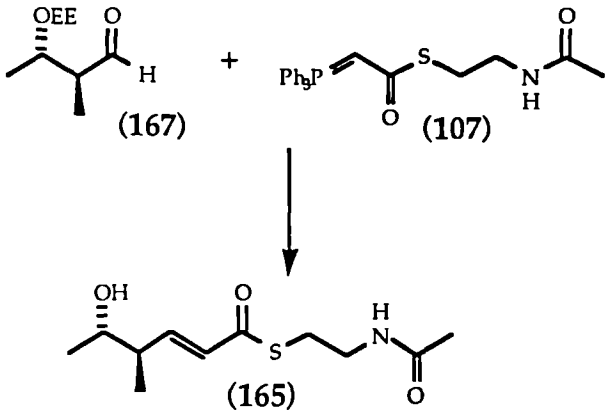


Scheme 3.1.31: Linear synthesis of the N-acetylcysteamine thioester of (2E, 4R, 5R)-5-hydroxy-4-methylhex-2-enoate.

Swern oxidation[53] of this produced the aldehyde (**167**) in 63% yield . The use of DIBAL to affect the direct conversion of the ester (**161**) (in protected form) to the aldehyde (**167**) was investigated, but proved to be unyielding.

A Wittig reaction[54] was used to produce producing ethyl (2E, 4S, 5S)-5-(1-ethoxy)ethoxy-4-methylhex-2-enoate **(168)** in 90% yield from the aldehyde (161). The free acid was formed by saponification which also led to deprotection upon acidification in 45% yield. This was followed by DCC DMAP[46] coupling with N-acetylcysteamine **(98)** to produce the N-acetyl cysteamine thioester (2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate **(165)** in 59% yield from the acid

A synthetic route of increased convergency was developed in order to reduce the amount of work required subsequent to putative label introduction. This involved reacting the aldehyde **(167)** with the ylid **(107)** as shown in scheme 3.1.32. Initial investigations were directed towards forming the ylid in situ from the triphenylphosphonium salt **(109)** in the presence of a base, but this approach proved unproductive



Scheme 3.1.32: Convergent synthesis of the N-acetylcysteamine thioester of (2E, 4R, 5R)-5-hydroxy-4-methylhex-2-enoate.

However, use of the preformed ylid **(107)**, as described in chapter 3, was found to affect the required conversion. Deprotection of the hydroxy function prior to purification led to recovery of the desired material **(165)** in 61% yield. This synthetic scheme was the one used for the preparation of the labelled material. The ^{1}H and ^{2}H nmr spectra of unlabelled and labelled materials are shown in figure 3.1.4.

3.1.10: Administration of putative biosynthetic precursors to *Pseudomonas fluorescens*

The biomimetic forms of the three proposed intermediates on the biogenetic pathway to pseudomonic acid **(44)** were synthesised in labelled

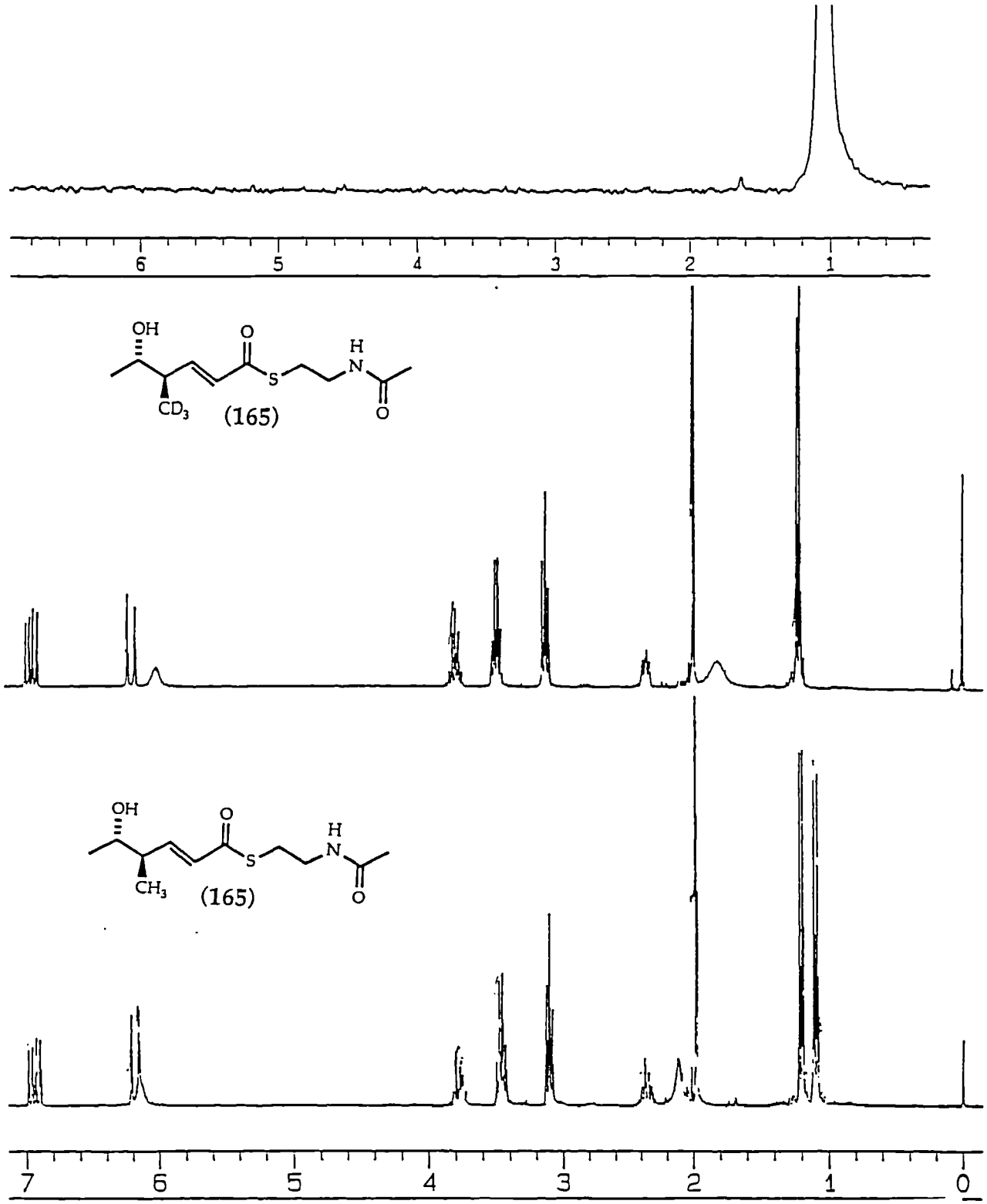


Figure 3.1.4: ^{1}H and ^{2}H nmr spectra of unlabelled and labelled N-acetylcysteamine thioester of(2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (165).

form as described. These were then administered to the bacteria simultaneously with tetradecylthiopropionic acid (89), the β-oxidase inhibitor. Cultures were worked up as usual and the isolated methyl pseudomonate (58) analysed by nmr spectroscopic methods.

The ^{13}C nmr spectrum produced from the isolated metabolite (12mg) after adminstration of 207mg of the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-acetoacetate (144) is shown in figure 3.1.5. There is no coupling between the C-13 and C-12 resonances indicating no intact incorporation. The resonance at δ51.51 appears to be weaker than the other signals in the spectrum. This is the peak due to the methyl ester introduced as part of the purification procedure, and its relative weakness may be the result of enhancement of all other signals via catabolism. Degradation of the administered compound would be expected to lead to both [1-^{13}C]- and [2-^{13}C]- acetates, so incorporation at all positions would be expected. The two carbons derived from methionine also appear weaker than the rest, though this is not so prominent as for the methyl signal.

The ^{2}H nmr spectrum resulting from the metabolite isolated (13mg) after administration of 243mg of [5-$^{2}H_3$]-(2S, 3S)-3-hydroxy-2-methyl butanoate (159) is shown in figure 3.1.6. The absence of a signal at δ0.95 indicates that intact incorporation did not take place. The signals observed at δ1.25, δ2.6 and δ3.7-4.3 indicate that catabolism has occurred. The signal at δ1.9 is probably due to the presence of D_2O. During the work-up 12mg of the administered compound was also isolated from the culture broths.

Figure 3.1.7 displays the ^{2}H nmr spectrum of the methyl pseudo monate that was isolated from the cultures to which 197mg of [7-$^{2}H_3$]-(2E,4S,5S)-5-hydroxy-4-methyl-hex-2-enoate (165) were administered. There is no evidence for intact incorporation of label, although the weak signals at δ1.22 may be indicative of some incorporation of label via catabolism.

3.2: THE ORIGIN OF C-15 OF MONIC ACID (84)

3.2.1: The cleavage of acetate

The derivation of C-15 from acetate as opposed to methionine[1] in monic acid (84) biosynthesis was the first discovery of such a process. As was mentioned in chapter 2, other examples have since been discovered.

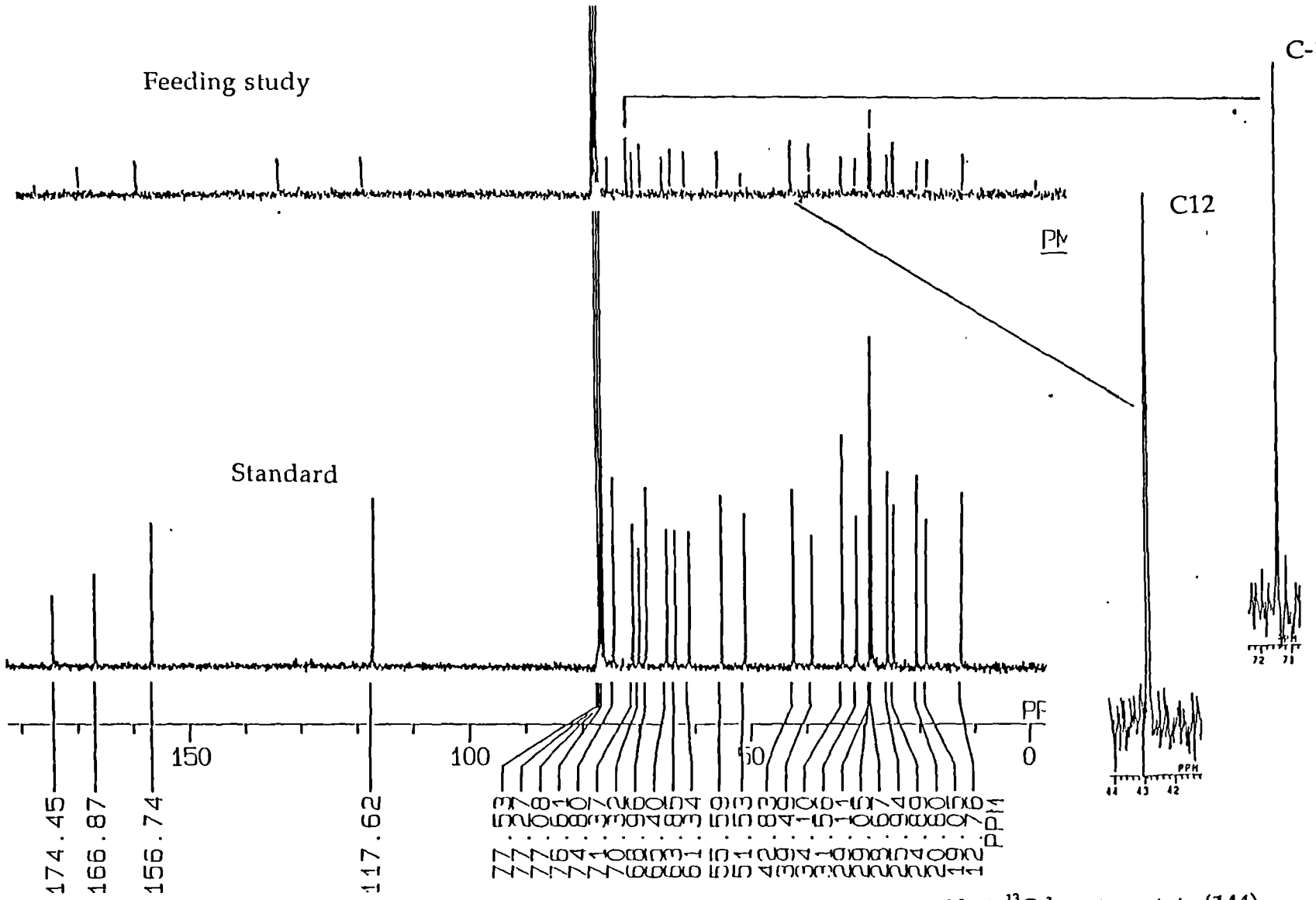


Figure 3.1.5: ^{13}C nmr spectrum of pseudomonic acid (**44**) after administration of [2,3-$^{13}C_2$]-acetoacetate (**144**).

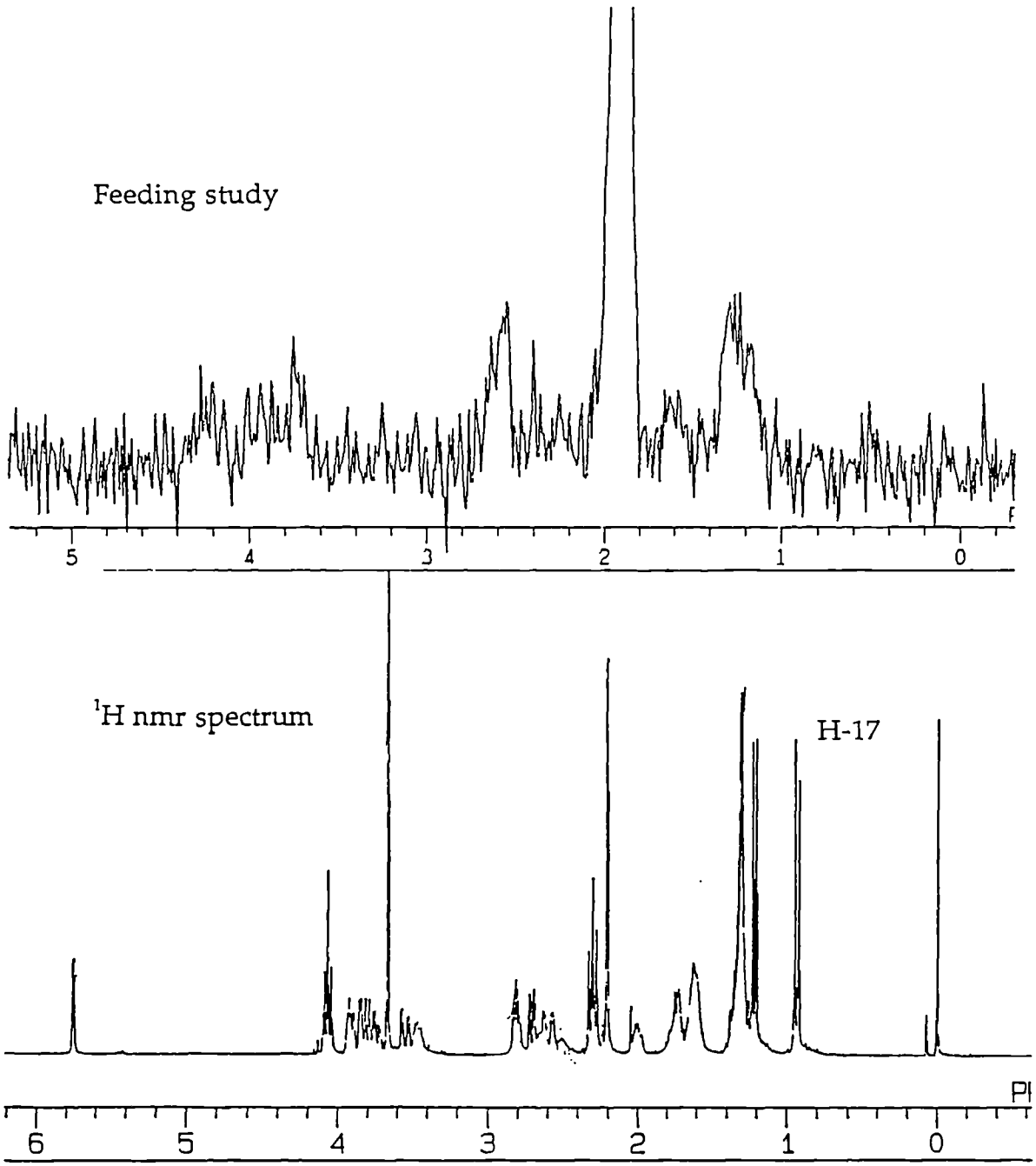


Figure 3.1.6: ^{2}H nmr spectrum of pseudomonic acid (44) after adminstration of [5-$^2H_3$]-(3S,2S)-3-hydroxy-2-methylbutanoate (159).

Feeding study

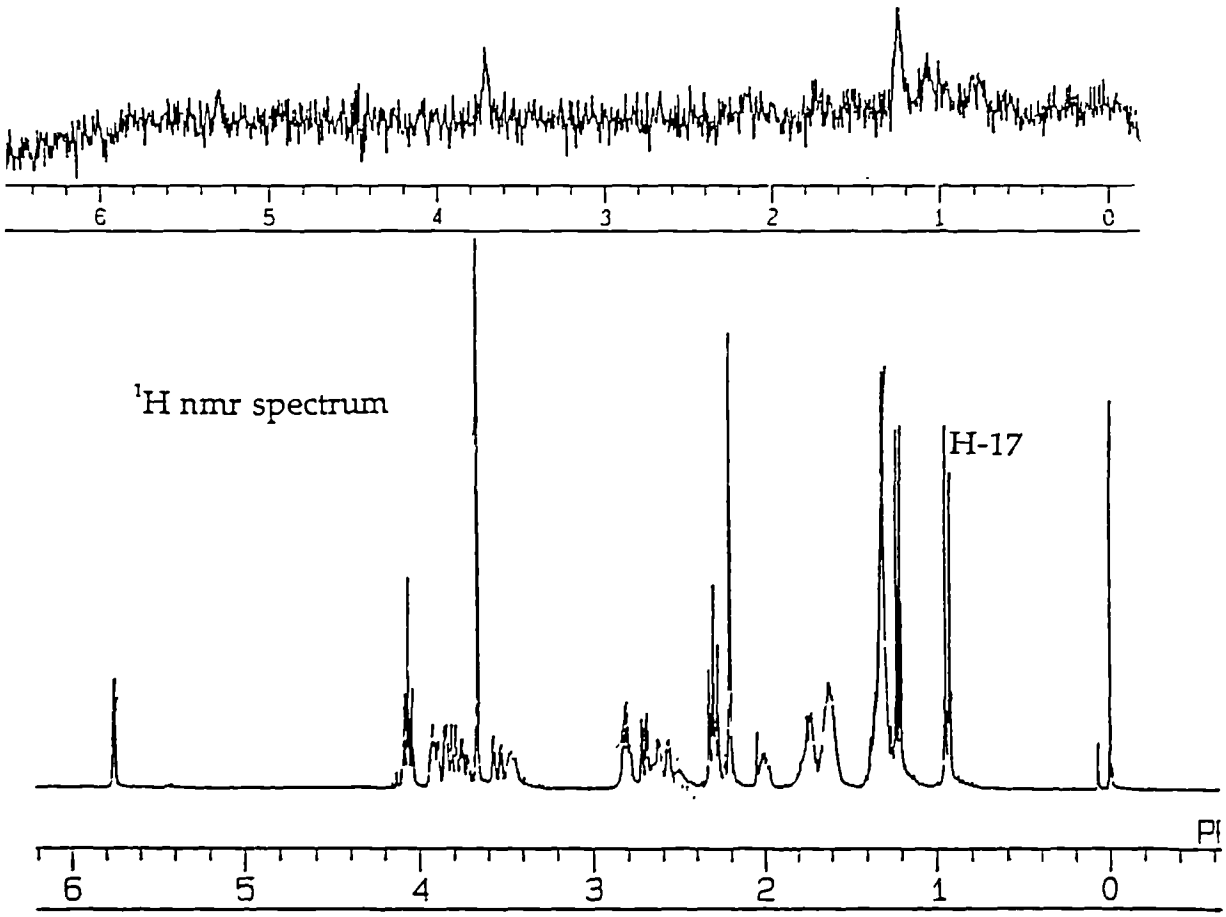
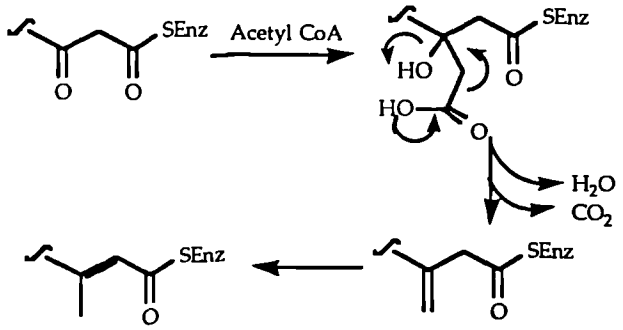


Figure 3.1.7: ^{2}H nmr spectrum of pseudomonic acid (44) after administration of [7-$^2H_2$]-(2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (165).

The explanantion of this phenomenon by way of β–hydroxy–β–methyl glutarate (64) has since been invalidated.

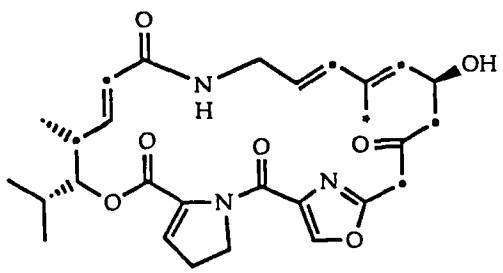
Studies on virginiamycin (60) biosynthesis gave rise to the suggestion that a similar pathway was being expressed.[55] This was rationalised by invoking the aldol condensation of an acetyl CoA (9) unit onto a pre-formed polyketide chain, followed by thioester hydrolysis, decarboxylative dehydration and alkene isomerisation, as shown in scheme 3.2.1.

Evidence for this was presented from feeding studies utilising [3-^{13}C]-serine (169). This is converted into acetyl CoA (9)[56], via pyruvate (11), and was postulated, therefore, to act as a delayed source of administered acetate.



Scheme 3.2.1: Mechanism for introduction of a cleaved acetate unit onto a polyketide chain.

When fed to *Streptomyces virginiæ* [2-^{13}C]-acetate gave an equal enrichment of all the carbons derived from this moiety in virginiamycin (60) biosynthesis. However, when [3-^{13}C]-serine (169) was similarly used, the signal due to C-33 (the branched methyl) was enhanced by ca2.2 times that of the other enriched signals, figure 3.2.1., as was predicted by the proposed pathway.[55]



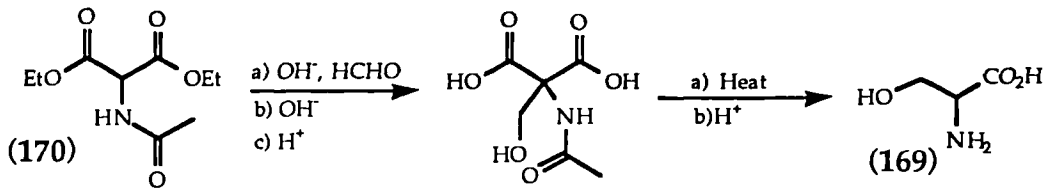
(60)

Figure 3.2.1: Enhanced enrichment of C-33 (*) by [3-^{13}C]-serine (167), compared to other positions labelled by [2-^{13}C]-acetate (•)

Interestingly no follow up to this work has been reported on either the biogenesis of virginiamycin (60) or any of the other metabolites shown to exhibit this behaviour. The possibility that such a pathway may be operating in the biosynthesis of pseudomonic acid (44) was therefore investigated.

3.2.2: The synthesis of labelled serine (169)

The most obvious label source for this synthesis was recognised as being [1-^{13}C] formaldehyde. A synthesis utilising this had already been described in the literature[57] and proved to be amenable for this purpose, scheme 3.2.2



Scheme3.2.2: Synthesis of serine (169)

Diethyl acetamidomalonate (170) was reacted with formaldehyde in the presence of a catalytic amount of sodium hydroxide. The mixture was subsequently treated with an excess of sodium hydroxide overnight, acidified with glacial acetic acid, heated to induce decarboxylation and treated with concentrated hydrochloric acid to remove the amine protecting group. Isolation and recrystallisation gave serine (169) in 61% yield.

3.2.3: The administration of serine to *Pseudomonas fluorescens.*

In order to quantify any differential enrichment at C-15 in the ^{13}C nmr spectrum of methyl pseudomonate (58), two experiments were run simultaneously. To one set of cultures was administered 250 mg of sodium [2-^{13}C]-acetate, whereas to a second set of cultures derived from the same seed flask (and thus the same slope) was fed 250mg of [3-^{13}C]-serine (169).

Unfortunately, the cultures fed with acetate failed to complete the growth phase after administration, despite commencement of production

having been observed by HPLC. The bacteria to which labelled serine (**169**) had been administered did complete the growth phase, but produced only 3mg of the metabolite which was not enough material to obtain an adequate ^{13}C nmr spectrum.

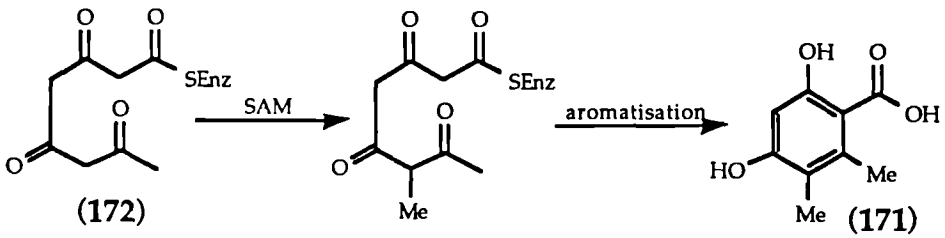
Therefore nothing can be ascertained from this work regarding the biosynthetic origins of C-15 on pseudomonic acid (**44**).

3.3: THE ORIGINS OF C-16 AND C-17

3.3.1: Biosynthetic methylation

That C-16 and C-17 were derived from the 'C_1-pool' via S-adenosyl methionine (**59**) was demonstrated during the initial investigations into pseudomonic acid (**44**) biosynthesis.[1] This is a well defined and general pathway for the introduction of methyl groups.[58] However, the timing of this process relative to other operations in biosynthetic pathways is not known.

Some evidence for methylation subsequent to chain extension was gained from investigations into the biosynthesis of 5-methylorsellinic acid (**171**), whereby tetraacetic acid (**172**) was inferred to be the methylation substrate,[59] scheme 3.3.1. However it was also possible to rationalise this process as occurring after release from the polyketide synthase, and thus being separate from the chain elongation process.

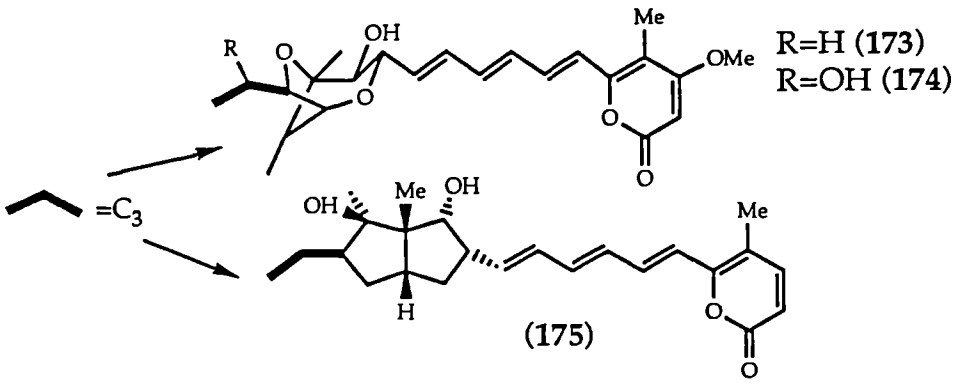


(**172**) (**171**)

Scheme 3.3.1: Methylation of tetraacetic acid (**172**) on the biosynthetic pathway to 5-methylorsellinic acid (**171**)

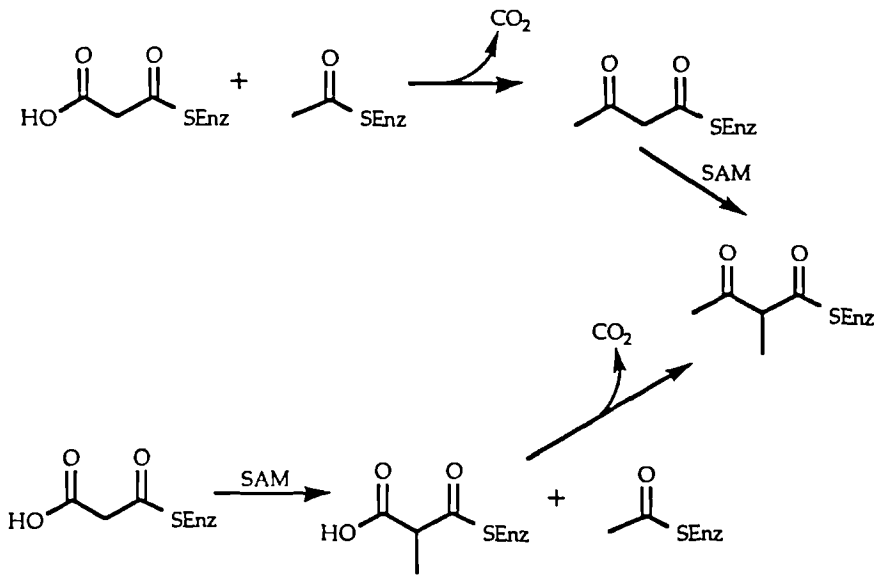
Study of the biosynthesis of aurovertin B (**173**) , aurovertin D (**174**) and asteltoxin (**175**) indicated a dual incorporation of propionate and acetate/ methionine moities into the C3 starter unit,[60] scheme 3.3.2. This result indicates that the methylation of acetate prior to chain incorporation may be a valid biosynthetic hypothesis.

The processive polyketide hypothesis predicts that methylation occurs contemporaneously to homologation, but not whether the process is prior or subsequent to the chain elongation step. In the former case, the chain extension unit, by analogy to macrolide biosynthesis, would be expected to be methylmalonate.



Scheme 3.3.2: The biosynthesis of the aurovertins (**173**, **174**) and asteltoxin (**175**) from a C3 starter unit derived from both propionate and acetate/methionine.

However if this is the case in pseudomonic acid (**44**) biosynthesis the methylmalonate would necessarily have to be formed by methylation of malonate, as opposed to carboxylation of propionate. This is summarised in scheme 3.3.3.

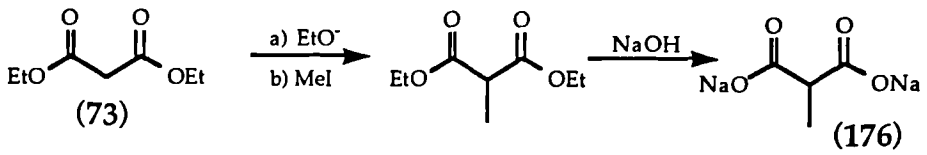


Scheme 3.3.3: Possible modes of methylation via S-adenosylmethionine (**59**)

In order to attempt clarification of the involvement of methylmalonate in polyketide biosynthesis it was decided to investigate the methylation processes in pseudomonic acid (**44**) biogenesis. For these studies a labelled form of methylmalonate was required.

3.3.2: Synthesis of labelled methylmalonate

The synthesis of labelled malonates has already been described in chapter two. Methylation of diethyl malonate (**73**) was possible via the use of sodium ethoxide and trapping of the resultant enolate with iodomethane, scheme 3.3.4. Any residual diethyl malonate was removed by a sodium hydroxide wash.[61] Distillation of the residue yielded diethyl methylmalonate in 81% yield. For administration purposes this was quantitatively saponified to the disodium salt (**176**).



Scheme 3.3.4: Synthesis of disodium methylmalonate (**176**)

3.3.3: Administration of labelled methylmalonate to *Pseudomonas fluorescens.*

In an initial experiment 250mg of disodium [4-^{13}C]-methylmalonate (**176**) was administered, leading to the isolation of 22mg of metabolite The ^{13}C nmr spectrum of this is shown in figure 3.3.1. No evidence for enhancement of signals can be observed at either δ12.76 (C-17) or δ65.37 (C-16).

For increased sensitivity, 250mg of [2,4-$^{13}C_2$]-methylmalonate (**176**), were fed to the bacteria and 25mg of metabolite was isolated. In the ^{13}C nmr spectrum of this (diagram3.3.2) there were no ^{13}C-^{13}C couplings detected between either the C-16/C8 or the C-17/C-12 resonances as would be expected had intact incorporation occurred. There was no evidence for any significant label incorporation into the rest of the metabolite.

This last fact was rather surprising as the vitamin B_{12} dependent methylmalonyl CoA (**177**) rearrangement to succinyl CoA (**178**)[62] shown in scheme 3.3.5 might have been expected to 'leak' label into pseudomonic acid (**44**) biosynthesis via the Krebs' cycle.

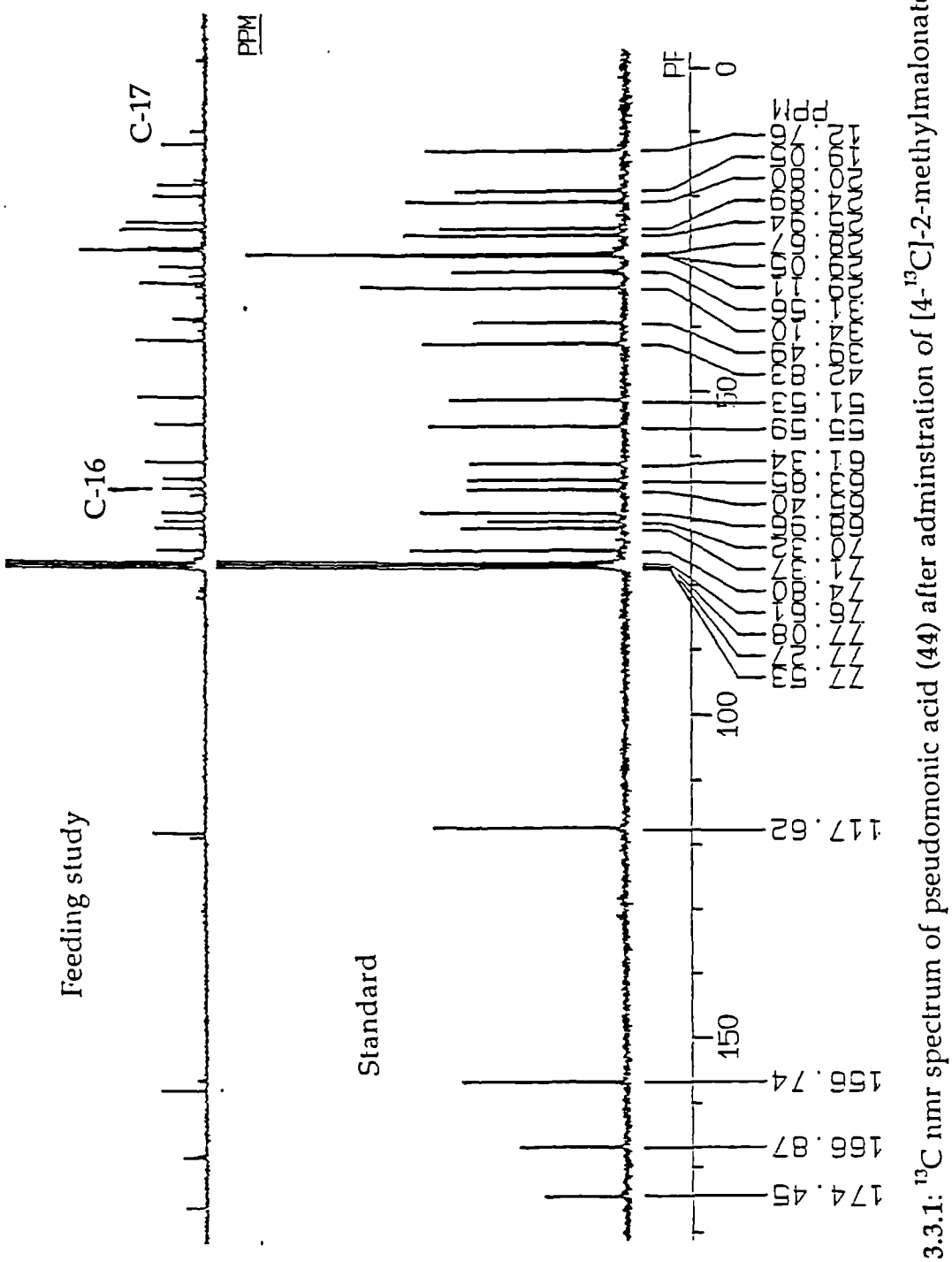


Figure 3.3.1: ^{13}C nmr spectrum of pseudomonic acid (44) after adminstration of [4-^{13}C]-2-methylmalonate.

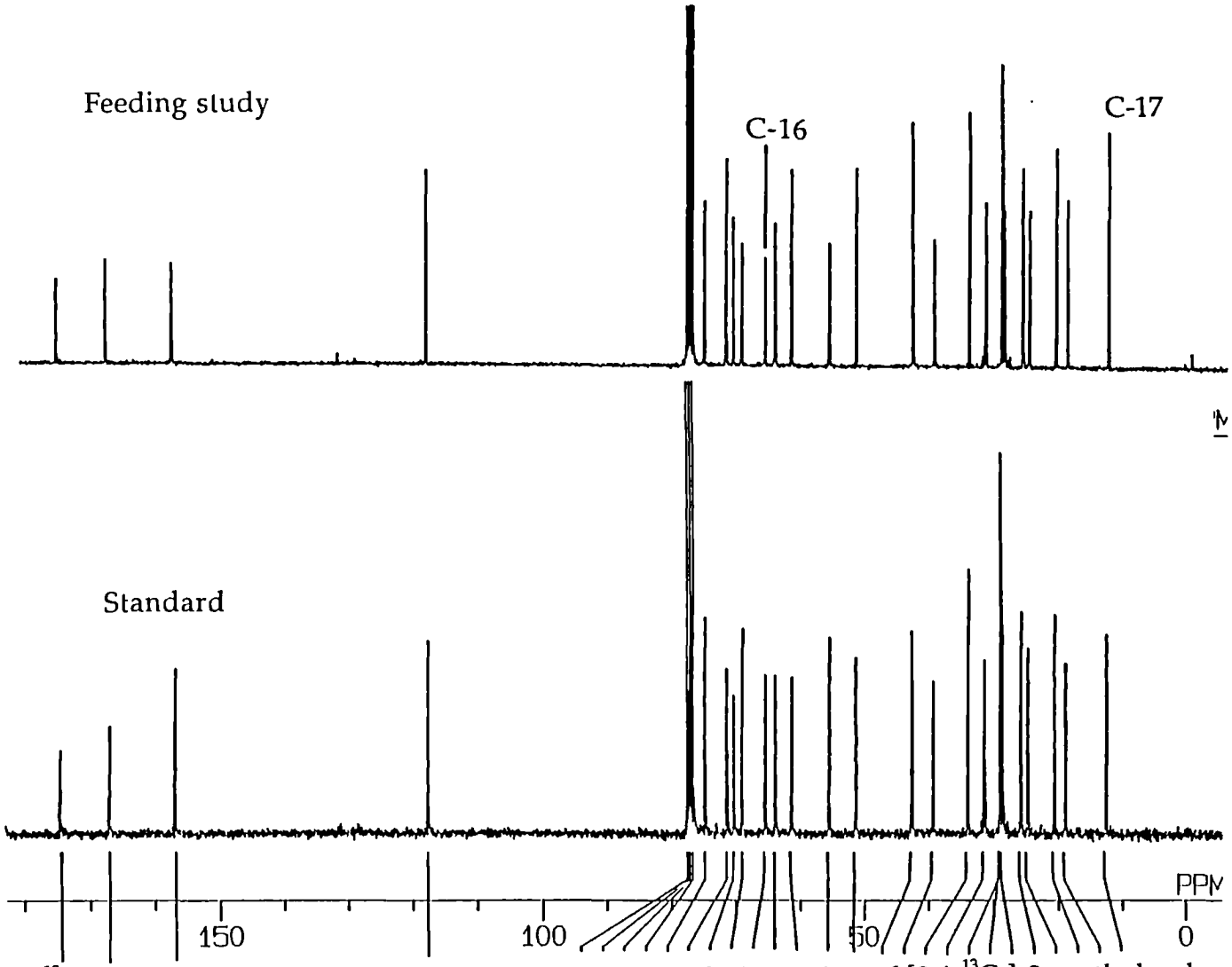
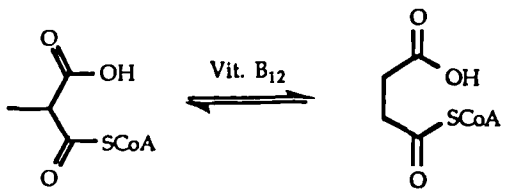


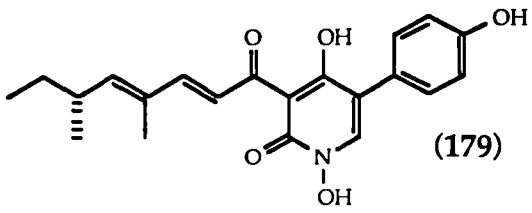
Figure 3.3.2: ^{13}C nmr spectrum of pseudomonic acid (44) after adminstration of [2,4-$^{13}C_2$]-2-methylmalonate.



(177) (178)

Scheme 3.3.5: The rearrangement of methylmalonyl CoA (**177**) to succinyl CoA (**178**)

A sample of [2,4-$^{13}C_2$] methylmalonate was supplied to O'Hagan and co-workers for similar studies into the biosynthesis of tenellin (**179**) in *Beauvaria bassiana*. Again, no specific incorporation of label into the methyl positions was observed, nor was there any evidence for non-specific incorporation of label.[63]



(179)

This work seems to supply strong evidence that methylation occurs after chain elongation onto a polyketide chain, in accordance with more classical ideas. The nature of the relationship between the methylase and the polyketide synthase remains unresolved, however. If pseudomonic acid (**44**) biosynthesis is mediated by a Type I polyketide synthase then it may be inferred that the methyltransferase activity will be found within the multifunctional protein.

3.4: Conclusions and further work.

From these studies it has been shown that methylation via the 'C1-pool' in polyketide biosynthesis occurs directly onto the polyketide chain, and not via pre-methylation of a chain extending unit. The question of methylation via cleavage of acetate units remains unanswered. The experiment using labelled serine needs to be repeated. The use of [2-^{13}C]-malonate, and variation in the time of feeding to see if any differential incorporation can be observed might also be useful for these studies. It is

plausible that malonate, via decarboxylation, is the actual source of acetate for the postulated aldol condensation reaction,

The results from feeding proposed chain elongation intermediates suggested that catabolism had taken place. Repetition of these experiments with increased amounts of inhibitor, or even the use of an alternative inhibitor, may prove worthwhile. Increased pulse feeding may also prove to be a beneficial approach.

However this problem appears to be endemic to studies of this kind and it is probable that full realisation of the biochemical processes underpinning polyketide biosynthesis will remain elusive until purification of a variety of polyketide synthases has been achieved.

EXPERIMENTAL

General experimental

The ^{2}H nmr spectra were run on a Jeol 400X instrument, at 61.3 MHz, in $CHCl_3$ solution with $C^{2}HCl_3$ as an external reference. Optical rotations were recorded on a Perkin-Elmer Z41MC polarimeter.

All other general experimental is as previously described.

Synthesis of ethyl acetoacetate (145)

To 15ml of freshly distilled anhydrous THF under a nitrogen atmosphere at -78°C was added 3.39g (21.07mmol) of HMDS. This was followed by the addition of 8.12ml (19.5mmol) of a 1.6M solution of butyl lithium. After 30 minutes ethyl acetate (1.03g, 11.07mmol) was added and the mixture left a further 30 minutes. Acetyl chloride (0.87g, 11,2mmol) was then added and the mixture left for 30 minutes. After being allowed to warm to room temperature 15ml of 2M HCl was added to the reaction and the layers separated. The aqueous layer was extracted with ethyl acetate (3 x) and the combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield an orange oil. This was purified by bulb to bulb distillation (46°C, 10mbar) to give **ethyl acetoacetate (145)** (1.0347g, 7.93mmol, 71%) as a colourless oil. υ_{max}(thin film) 1721 cm^{-1}; δ_H 1.3 (3H, t, J=6.5Hz CH_3), 2.3 (3H, s, CH_3CO), 3.45 (2H, s, $COCH_2CO$), 4.15 (2H, q, J=6.5Hz, OCH_2); m/z: 131 (M^++1, 4.5%) 130 (m^+, 3.7), 85 (15.4), 84 (16.1), 44 (100).

Synthetic approaches to the N-acetylcysteamine thioester of acetoacetate (144).

A) Via carbonyl activation (The method of Martin[33])

a) Ethyl acetoacetate (145) (10g, 77mmol) was dissolved in 50ml of dry toluene in a Dean-Stark apparatus under nitrogen. To this was added 20mg of PTSA and 10.79ml (15.4mmol)) of ethylene glycol. The mixture was heated under reflux overnight. After cooling the solution was washed sequentially with 50ml of 5% sodium bicarbonate and water (2 x 50ml). The organics were then dried, filtered and concentrated to yield **ethyl (3,3-ethyleneketal)butanoate (146)** as a colourless oil (8.977g, 5.196mmol, 67%).

δ_H (60MHz) 1.2 (3H, t, J=7Hz, CH_3), 1.45 (3H, s, CH_3), 2.65 (2H, s, CH_2CO), 3.95 (4H, s, $O(CH_2)_2O$), 4.15 (2H, q, J=7Hz, OCH_2)

b) To 1g (5.75mmol) of ethyl (3,3-ethyleneketal)butanoate (**146**) was added 3.15ml of 2M sodium hydroxide solution. The mixture was left stirring overnight. The water was removed *in vacuo* and the residue freeze dried to yield **sodium (3,3-ethyleneketal)butanoate (147)** as a white solid (0.86g, 5.75ml, 100%). δ_H(60MHz, D_2O) 1.5 (3H, s, CH_3), 2.55 (2H, s, CH_2CO), 4.0 (4H, s, $O(CH_2)_2O$)

c) The sodium salt (**147**) (0.697g, 4.2mmol) was suspended in 5ml of freshly distilled anhydrous THF. To this was added methyl chloroformate (0.99g, 10.5 mmol) and 0.051g (0.42mmol) of DMAP. The mixture was left vigorously stirring overnight before being filtered through celite. The filtrate was washed with saturated ammonium chloride, dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give the **methyl formate-(3,3-ethylene ketal) butanoate mixed anhydride (148)** (0.525g, 2.574 mmol, 61%). δ_H(60 MHz) 1.5 (3H, s, CH_3), 2.6 (2H, s, CH_2), 3.9 (3H, s, CH_3), 4..0 (4H, s, $O(CH_2)_2O$)

d) This mixed anhydride (**148**) (0.648g, 3.18mmol) was dissolved in 8ml of dry THF to which was added freshly prepared N-acetylcysteamine (1.02g, 6.41mmol). After stirring overnight 10ml of ethyl acetate was added and the solution washed with 1M potassium hydroxide solution. The aqueous layer was extracted with ethyl acetate (20ml) and the combined organic extracts were dried over anhydrous magnesium sulphate, filtered and concentrated to give 0.625g (2.33mmol, 80%) of the **N-acetylcysteamine thioester of (3,3-ethylene ketol)butanoate.** δ_H (60 MHz) 1.4 (3H, s, CH_3), 2.05 (3H, s, CH_3), 2.95 (2H, s, CH_4CO), 3.05 (2H, t, J=6.2Hz, CH_2S), 3.55 (2H, m, CH_2N), 4.0 (4H, s, $O(CH_2)_2O$), 6.15 (1H, br s, NH)

B Via Meldrum's acid (The method of Ley[35])

a) Meldrum's acid (**153**) (1.805g, 12.6mmol) was added to 40ml of freshly distilled dichloromethane under a nitrogen atmosphere. To this

was added 1.09ml (15.12 mmol) of acetyl chloride and 1.18ml (24.5mmol) of pyridine. The resultant mixture was stirred for an hour at 0°C and then for a further hour at room temperature. The mixture was then poured into 60ml of 2M HCl at 0°C. The layers were separated and the aqueous layer extracted with dichloromethane (2 x 50ml). The combined organics were then dried over magnesium sulphate, filtered and concentrated *in vacuo* to yield 2.179g (11.72 mmol, 93%) of **acetyl Meldrum's acid (148)** as orange-brown crystals. δ_H 1.83 (6H, s, 2 x CH_3), 2.8 (3H, s, CH_3), 15.05 (1H, br s, OH)

b) The acetyl Meldrum's acid (**148**) (0.107g, 0.575mmol) was dissolved in 8ml of chloroform under a dry nitrogen atmosphere. Freshly prepared N-acetylcysteamine (0.680g, 5.75mmol) was added in 2ml of chloroform. The mixture was heated under reflux and monitored by TLC. After 16 hours no apparent change had been observed. An aliquot was concentrated and analysed by ^1H nmr spectroscopy, which revealed the presence of starting materials only. Prolonged reaction times gave no further change.

C) Via N,S-diacetylcysteamine (The method of Cane[34])

To 40ml of freshly distilled anhydrous THF under a dry nitrogen atmosphere at -78°C was added 0.72ml (5.126mmol) of diisopropylamine. To this was added 3.21 ml of a 1.6M solution of n-butyl lithium (5.126mmol). After 20 minutes N,S-diacetylcysteamine (**151**) (0.413g, 2.563mmol) was added as a solution in 10ml of anhydrous THF. After a further 20 minutes was added acetyl chloride (0.18ml, 2.563mmol) in a dropwise manner. After 5 minutes the mixture was allowed to warm to room temperature and then washed with water (40ml) and brine (30ml). The organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*.

The crude product was shown by ^1H nmr to be returned N,S-diacetyl cysteamine.

D) Via acetoacetic acid

a) Preparation of acetoacetic acid (156)

To ethyl acetoacetate (145) (4.222g, 32.5 mmol) was added 25ml of an aqueous 2M solution of sodium hydroxide. This was stirred at 5-10°C for 7 hours before being cooled to 0°C. The pH was adjusted to 1.0 by the slow addition of 6M HCl and the resultant solution extracted with cold ethyl acetate (10 x 30ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give **acetoacetic acid (156)** (3.095g, 30.34mmol, 93%) as a colourless oil that froze to a white solid upon refrigeration. υ_{max}(thin film) 2930, 1712 cm^{-1}; δ_H 2.3 (3H, s, CH_3CO), 3.5 (2H, s, $COCH_2CO$), 5.05 (s, enol CH), 10.05 (1H, br s, OH); δ_C 30.05 (CH_3), 49.09 (CH_2), 171.77 (CO), 201.77 (CO_2H)

b) Attempted thioesterification mediated by phenyl dichlorophosphate

Acetoacetic acid (156) (0.25g, 2.4mmol) was dissolved in 5ml of DME at 0°C. To this was added sequentially pyridine (0.41ml, 5mmol), N-acetylcysteamine (98) (0.89g, 7.48mmol) and phenyl dichlorophosphate (157) (0.56ml, 3.75mmol). The mixture was allowed to warm to room temperature with stirring overnight. The resultant suspension was filtered through celite and concentrated to leave a yellow oil.

The ^1H nmr spectrum of this gave no evidence for formation of product

c) Attempted thioesterification mediated by DCC/DMAP

To a stirred solution of acetoacetic acid (156) (0.338g, 3.32mmol) and N-acetylcysteamine (98) (0.51g, 4.32mmol) in dichloromethane at 0°C under nitrogen was added DCC (0.72g, 3.49mmol) and DMAP (0.043g, 0.349mmol) slowly in 5ml of dichloromethane. The mixture was left overnight and then filtered through celite. The filtrate was dried over anhydrous magnesium sulphate, filtered and concentrated to leave a yellow oil.

The ^1H nmr spectrum of this showed no evidence for formation of product.

d) Thioesterification mediated by DCC/pyridine

To acetoacetic acid (**156**) (0.235g, 2.3 mmol) in 10ml of dichloromethane at -25°C under a nitrogen atmosphere was added freshly prepared N-acetyl cysteamine (**156**) (0.356g, 2.99mmol) in 5ml of dichloromethane. After five minutes was added DCC(0.498, 2.42mmol) in dichloromethane containing 3 drops of pyridine. The system was allowed to warm to room temperature overnight and the resultant suspension filtered through celite. The filtrate was dried over anhydrous magnesium sulphate, filtered, concentrated. Purification by preparative TLC using plates pre-treated with triethyl amine (acetone/dichloromethane, 2:3) gave the **N-acetylcysteamine thioester of acetoacetate (156)** as a colourless syrup (0.355g, 1.635mmol, 71%). $\upsilon_{max}(CHCl_3)$ 3390, 1719, 1694, 1668, 1651cm^{-1}; δ_H 1.97 (3H, CH_3CON), 2.28 (3H, s, CH_3CO), 3.09 (2H, t, J=6.7Hz, CH_2S), 3.45 (2H, m, CH_2N), 3.73 (2H, s, $COCH_2CO$), 5.45, (s, enol CH), 6.37 (1H, br s, NH); δ_C 23.14 (CH_2N), 29.21 (CH_2S), 30.45 (CH_3CO), 39.15 (CH_3CON), 58.03 (CH_2), 170.55 (CON), 192.26 (CO), 199.95 (COS); m/z 203 (M^+, 0.6%), 119 (36.5), 118 (11.5), 85 (16.7), 60 (84), 43 (97.7), 30 (100).

Synthesis of the N-acetylcysteamine thioester of [2,3-$^{13}C_2$] acetoacetate (144)

a) To 15ml of freshly distilled anhydrous THF under a nitrogen atmosphere at -78°C was added 2.45g (15.19mmol) of HMDS. This was followed by the addition of 5.91ml (14.77mmol) of a 1.6M solution of butyl lithium. After 30 minutes ethyl [2-$^{13}C_2$]-acetate (0.76g, 8.44mmol) was added and the mixture left a further 30 minutes. [1-^{13}C]-acetyl chloride (0.826g, 10.13mmol) was then added and the mixture left a further 30 minutes. After being allowed to warm to room temperature 15ml of 1M HCl was added to the reaction and the layers separated. The aqueous layer was extracted with ethyl acetate (3 x 20ml) and the combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield an orange oil. This was purified by bulb to bulb distillation (46°C, 20 mmHg) to give **ethyl [2,3-$^{13}C_2$]-acetoacetate (145)** (0.763g, 5.82mmol, 69%) as a colourless oil. υ_{max} 1722 cm^{-1}; δ_H 1.29 (3H, t, J=7.1Hz, CH_3), 2.27 (3H, dd, $^2J_{CH}$=6.2Hz, $^3J_{CH}$=1.3Hz, $CH_3{}^{13}CO$), 3.45 (2H, dd, $^1J_{CH}$=130.2Hz, $^2J_{CH}$=6.2Hz, $^{13}CH_2{}^{13}CO$), 4.17 (2H, q, J=7.1Hz, CH_2); m/z 133 (M^++1, 5.8%), 132 (M^+, 5.0), 131 (3.1), 87 (14.3), 86 (18.9), 44 (100).

b) To ethyl [2,3-$^{13}C_2$]-acetoacetate (**145**) (0.753g, 5.71 mmol) was added 5ml of an aqueous 2M solution of sodium hydroxide. This was stirred at 5-10°C for 7 hours before being cooled to 0°C. The pH was adjusted to 1.0 by the slow addition of 6M HCl and the resultant solution extracted with cold ethyl acetate (10 x 10ml). The combined organic layers were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give **[2,3-$^{13}C_2$]-acetoacetic acid (156)** (0.436g, 4.1mmol, 72%) as a colourless oil that froze to a white solid upon refrigeration. υ_{max}(thin film) 2933, 1709 cm^{-1}; δ_H (3H, d $^2J_{CH}$=6.4Hz, $CH_3{}^{13}CO$), 3.5 (2H, dd, $^1J_{CH}$=130.5Hz ,$^2J_{CH}$=6.4Hz, $^{13}CH_2{}^{13}CO$)

c) To [2,3-$^{13}C_2$]-acetoacetic acid (**156**) (0.428g, 4.04 mmol) in 5ml of dichloromethane at -25°C under a nitrogen atmosphere was added freshly prepared N-acetylcysteamine (**98**) (0.626g, 15.24mmol) in 2ml of dichloro methane. After five minutes was added DCC (8.70, 4.24mmol) in 2ml of dichloromethane containing 3 drops of pyridine. The system was allowed to warm to room temperature overnight and the resultant suspension filtered through celite. The filtrate was dried over anhydrous magnesium sulphate, filtered, concentrated. Purification by prep. TLC using plates pre-treated with triethyl amine (acetone/dichloromethane, 2:3) gave the **N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-acetoacetate (144)** as a colourless syrup (0.331g, 1.80mmol, 45%). υ_{max}($CHCl_3$) 3392, 1719, 1693, 1668, 1651 cm^{-1}; δ_H 1,97 (3H, s, CH_3CON), 2.28 (3H, dd, J^2_{CH}=6.3Hz J^3_{CH}=1.1Hz CH_3CO), 3.09 (2H, t, J=6.6Hz, CH_2S), 3,35 (3H, m, CH_2N and highfield resonance of $^{13}CH_2$ doublet), 3.73 (2H, dd, $^1J_{CH}$=130.7Hz $^2J_{CH}$=6.3Hz, $^{13}CH_2$), 5.94 (1H, br s, NH); δ_C 58.3 (d, J=37.6 Hz, $^{13}CH_2$), 199.95 (d, J=37.6 Hz, ^{13}CO); m/z 205 (M^+, 0.8%), 119 (41,3), 118 (9.7), 87 (15.3), 44 (56.7), 43 (43.4), 30 (100).

Synthesis of the N-acetylcysteamine thioester of (2S, 3S)-3-hydroxy-2-methyl butanoate (159)

a) To 40g of D-glucose (**10**) in a 500ml conical flask was added 300ml of water. After dissolution 20 g of dried baker's yeast (single strain, *Sacchoromyces cerevisiæ*) was added and the mixture shaken at 26°C (250 rpm) for 30 minutes. At this point 1g of ethyl acetoacetate (**145**) was added. A further 1g was added 12 hours later. After a further 36 hours the mixture was centrifuged (10,000g, 4°C, 10 minutes) and the supernatant extracted

with ethyl acetate (5 x 200ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* and the residue was purified by bulb to bulb distillation (51°C, 3mmHg) to give **ethyl (3S)-3-hydroxybutanoate (160)** (1.138g, 8.62mmol, 56%) as a colourless oil. $[\alpha]_D$=+33.64°, c=3.38, $CHCl_3$ (lit[65]: $[\alpha]_D$=+37.2°, c=1.3, $CHCl_3$); υ_{max}(thin film) 3456, 1739 cm^{-1}; δ_H 1.22 (3H, d, J=6.4Hz, CH_3CH), 1.28 (3H, t, J=7.2Hz, CH_3CH_2), 2.46 (2H, m, CH_2CO), 4.16 (2H, q, J=7.2 Hz, CH_2O), 4.19 (1H, m, CH_3CH); δ_C 14.2, 22.3, 42.7, 60.8, 64.1, 172.9; m/z 132 (M^+, 0.7%), 131 (M^+-1, 8.3), 117 (39.9), 102 (84), 88 (52.5), 87 (52.3)

b) To 20ml of freshly distilled anhydrous THF under a dry nitrogen atmosphere at -78°C was added 1.08ml of diisopropylamine (14.75mmol) followed by 9.22ml of a 1.6M solution of butyl lithium in hexanes (14.75). After 30 minutes 1g of ethyl (3S)-3-hydroxybutanoate (160) was added as a solution in 2ml of THF. After 20 minutes the mixture was warmed to -45°C for 30 minutes and then recooled to -78°C. Iodomethane (1.12g, 14.85mmol) was added and the mixture left to warm to room temperature overnight. To this was added 15ml of saturated ammonium chloride solution. The layers were separated and the aqueous layer extracted with ether (5 x 20ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The residue was purified by bulb to bulb distillation (56°C, 4mmHg) to give **ethyl (2S, 3S)-3-hydroxy-2-methyl butanoate (161)** as a colourless oil (0.764g, 5.24mmol, 71%). $[\alpha]_D$=+22.3°, c=1.7, $CHCl_3$ (lit[66]: $[\alpha]_D$=+19.1°, c=1.3, $CHCl_3$); υ_{max}(thin film) 3347, 1715 cm^{-1}; δ_H 1.15 (3H, t, 6.3Hz, CH_3CH_2), 1.25 (3H, d, J=6.4Hz, CH_3CH), 1.30 (3H, d, J=6.4Hz, CH_3CH), 2.45 (1H, dq, J=6.3 Hz, 6.4Hz, **H-2**), 3.85 (1H, m, **H-3**), 4.18 (2H, q, J=6.3Hz, OCH_2); δ_C 13.68 (CH_3), 14.21 (CH_3), 20.57 (CH_3), 47.21 (CHCO), 60.53 (CO_2CH_2), 69.33 (CHOH), 175.83 (CO_2); m/z 145 (M^+, 0.7%), 131 (7), 102 (84.2), 101 (36.5), 74 (100), 56 (48.3)

c): To 1.985g (13.6mmol) of ethyl (2S, 3S)-3-hydroxy-2-methylbutanoate (161) was added 15ml of 2M NaOH. The mixture was stirred overnight and then extracted with ethyl acetate (10ml). The pH of the aqueous layer was adjusted to 1.0 and then re-extracted with ethyl acetate (5 x 15ml). The combined organic extracts were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to leave 1.396g (11.83mmol,

87%) of **(2S, 3S)-3-hydroxy-2-methylbutanoic acid.** υ_{max}(thin film) 3393, 1717 cm^{-1}; δ_H 1.25, d, J=6.5Hz, CH_3), 1.29 (3H, d, J=6.5Hz, CH_3), 2.51 (1H, m, **H-2**), 3.87 (1H, m, CHOH), 6.8 (2H, br s, 2 x OH)

d) To 0.585g (4.94mmol) of (2S, 3S)-3-hydroxy-2-methylbutanoic acid in 5ml of dichloromethane under a nitrogen atmosphere at 0°C was added 0.764g (6.4mmol) of freshly prepared N-acetylcysteamine (**98**) in 2ml dichloromethane. To this was added DCC (1.067g, 5.19mmol) and DMAP (0.06g, 0.52mmol) in 5ml of dichloromethane The reaction was left to warm to room temperature overnight at which point the mixture was filtered through celite. The filtrate was washed with saturated ammonium chloride solution, dried over anhydrous magnesium sulphate, filtered and concentrated. The residue was purified by flash column chromatography (acetone/dichloromethane, 2:3 with 3 drops of triethylamine) to yield the **N-acetylcysteamine thioester of (2S, 3S)-3-hydroxy-2-methylbutanoate (159)** as a colourless syrup (0.841g, 3.85mmol, 78%). υ_{max}($CHCl_3$) 3299, 1686, 1657, 1562, 1557 cm^{-1}; δ_H 1.2 (3H, d, J=6.9Hz, CH_3), 1.24 (3H, d, J=6.5Hz, CH_3), 1.97 (3H, s, CH_3CO), 2.7 (1H, m, $CHCH_3$), 3.05 (2H, t, J=6.5Hz, CH_2S), 3.47 (2H, m, CH_2N), 3.96 (1H, m, CHOH), 5.8 (1H, br s, NH); δ_C 21.06 (CH_3), 22.41 (CH_3), 23.19 (CH_2N), 28.58 (CH_2S), 39.39 (CH_3CON), 55.7 (CHCO), 69.89 (CHOH), 170.5 (CON), 204.2 (COS); m/z: 220 (M^++1, 0.9%), 119 (100), 101 (11.3), 60 (83)

Synthesis of the N-acetylcysteamine thioester of [5-$^2H_3$]-(2S, 3S)-3-hydroxy-2-methylbutanoate (159).

a) To 15ml of freshly distilled anhydrous THF under a dry nitrogen atmosphere at -78°C was added 1.04g of diisopropylamine (10.33mmol) followed by 4.55ml of a 1.6M solution of butyllithium in hexanes (11.36mmol). After 30 minutes 0.606g (43.593mmol) of ethyl (3S)-3-hydroxybutanoate (**160**) was added as a solution 2ml of THF. After 20 minutes mixture was warmed to -45°C for 30 minutes and then recooled to -78°C. To the mixture [$^2H_3$]-iodomethane (1g, 6.89mmol) was added and the mixture left to warm to room temperature overnight. To this was added 15ml of saturated ammonium chloride solution. The layers were separated and the aqueous layer extracted with ether (5 x 20ml). The combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The residue was purified by bulb to

bulb distillation (56°C, 4mmHg) to give **ethyl [5-$^2H_3$]-(2S, 3S)-3-hydroxy-2-methylbutanoate (161)** as a colourless oil (0.462g, 3.082mmol, 67%). υ_{max}(thin film) 3352, 1715 cm^{-1}; δ_H 1.15 (3H, t, J=6Hz, CH_2CH_3) 1.30 (3H, d, J=6.5 Hz, CH_3), 2.45 (1H, br m, CHC^2H_3), 3.85 (1H, m ,CHOH), 4.18 (2H, q, J=6Hz, OCH_2); m/z: 134 (M^+-15, 5.0%), 105 (79.7), 104 (45.0), 77 (100), 59 (51.7).

d) To 0.443g (2.953mmol) of ethyl [5-$^2H_3$]-(2S, 3S)-3-hydroxy-2-methyl butanoate (**161**) was added 3ml of 2M NaOH. The mixture was stirred overnight and then extracted with ethyl acetate (10ml). The pH of the aqueous layer was adjusted to 1.0 and then re-extracted with ethyl acetate (5 x 15ml). The combined organic extracts were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to leave 0.285g (2.36mmol, 80%) of **[5-$^2H_3$]-(2S,3S)-2-3hydroxy-2-methyl butanoic acid.** υ_{max}(thin film) 3389, 1719 cm^{-1}; δ_H 1.29 (3H, d, J=6.5Hz, CH_3), 2.51 (1H, br m, CHC^2H_3), 3.87(1H, m, CHOH), 6.9 (2H, br s, 2 x OH)

c): To 0.276g (2.28mmol) of [5-$^2H_3$]-(2S, 3S)-3-hydroxy-2-methylbutanoic acid in 5ml of dichloromethane under a nitrogen atmosphere at 0°C was added 0.353g (2.97mmol) of freshly prepared N-acetylcysteamine (**98**) in 2ml dichloromethane. To this was added DCC (0.494g, 2.40mmol) and DMAP (0.03g, 0.24mmol) in 5ml of dichloromethane The reaction was left to warm to room temperature overnight at which point the mixture was filtered through celite. The filtrate was washed with saturated ammonium chloride solution, dried over anhydrous magnesium sulphate, filtered and concentrated. The residue was purified by flash column chromatography (acetone/dichloromethane, 2:3 with 3 drops of triethylamine) to yield the **N-acetylcysteamine thioester of [5-$^2H_3$]-(2S,3S)-3-hydroxy-2-methyl butanoate (159)** as a colourless syrup (0.299g, 1.35mmol, 59%). υ_{max}($CHCl_3$) 3303, 1687, 1656, 1562, 1558 cm^{-1}; δ_H 1.24 (2H, d, J=7Hz, CH_3), 1.97 (3H, s, CH_3CON), 2.70 (1H ,br m, CHC^2H_3), 2.83 (1H, br s, OH), 3.05 (2H,t, J=6.5Hz, CH_2S), 3.47 (2H, m, CH_2N), 3.96 (1H, m, CHOH), 6.42 (1H, br m, NH); δ_C 21.19 (CH_3), 23.32 (CH_2N), 28.74 (CH_2S), 39.44 (CH_3CON), 55.84 (CHCO), 70.04 (CHOH), 170.47 (CON), 204.17 (COS); δ_D 1.13 (br s); m/z 207 (M^+-15, 3.6%), 190 (2.5), 119 (100), 86 (40), 60 (86)

Mosher's ester of ethyl 3-hydroxybutanoate (164)

To 0.054g (0.41mmol) of racemic ethyl 3-hydroxybutanoate **(160)** was added 0.103g (0.41mmol) of R-(+)-Mosher's acid chloride, 5 drops of carbon tetrachloride followed by 5 drops of pyridine. The mixture was left to stand overnight. After this time was added 1ml of water, and the resultant mixture poured into 20 ml of ether. This was washed with 1M HCl , saturated sodium carbonate solution and water. The organic layer was dried over anhydrous magnesium sulphate, filtered and concentrated. Purification by preparative TLC (ethyl acetate/petroleum 40-60, 3:2) gave the **Mosher's ester of racemic ethyl 3-hydroxybutanoate (164)** (0.021g, 0.19mmol, 46%) as a colourless oil. δ_H 1.17-1.25 (3H, m, CH_3), 1.3-1.45 (3H, dd, J=18Hz, 7Hz, CH_3), 2.47-2.8 (2H, m, **H-2α, H-2β**), 3.45 (3H, s, **OMe**), 4.0-4.2 (2H, m, OCH_2), 5.57 (1H, m, **H-3**), 7.40, 7.54 (5H, 2 x br s, **Phenyl**); m/z 303 (M^+-45, 3%), 216 (2), 189 (100), 115 (24.3), 73 (34.3), 69 (34.8).

Mosher's ester of ethyl (3S)-3-hydroxybutanoate (162)

To 0.032g (0.24mmol) of ethyl (3S)-3-hydroxybutanoate **(160)** was added 0.061g (0.24mmol) of R-(+)-Mosher's acid chloride, 5 drops of carbon tetrachloride followed by 5 drops of pyridine. The mixture was left to stand overnight. After this time was added 1ml of water, and the resultant mixture poured into 20 ml of ether. This was washed with 1M HCl , saturated sodium carbonate solution and water. The organic layer was dried over anhydrous magnesium sulphate, filtered and concentrated. Purification by preparative TLC (ethyl acetate/petroleum 40-60, 3:2) gave the **Mosher's ester of ethyl (3S)-3-hydroxybutanoate (162)** (0.048g, , 0.13mmol, 54%) as a colourless oil. δ_H 1.19 (3H, t, J=7.1Hz, CH_3), 1.43 (3H, d, J=6.2Hz, CH_3), 2.48-2.73 (2H, m, **H-2α, H-2β**), 3.55 (3H, s, **OMe**), 4.05 (2H, m, OCH_2), 5.57 (1H, m, **H-3**), 7.40,7.52 (5H, 2 x br s, **phenyl**); m/z 303 (M^+-45, 2.2%), 216 (4.4), 189 (100), 115 (19.3), 73 (30), 69 (28,1).

Mosher's ester of ethyl (2S, 3S)-3-hydroxy-2-methylbutanoate (164)

To 0.054g (0.37mmol) of ethyl (2S,3S)-3-hydroxy-2-methylbutanoate (161) was added 0.093g (0.37mmol) of R-(+)-Mosher's acid chloride, 5 drops of carbon tetrachloride followed by 5 drops of pyridine. The mixture was left to stand overnight. After this time was added 1ml of water, and the resultant mixture poured into 20 ml of ether. This was washed with 1M

HCl , saturated sodium carbonate solution and water. The organic layer was dried over anhydrous magnesium sulphate, filtered and concentrated. Purification by preparative TLC (ethyl acetate/petroleum 40-60, 3:2) gave the **Mosher's ester of ethyl (3S,2S)-3-hydroxy-2-methylbutanoate (163)** (0.062g, 0.17mmol, 46%) as a colourless oil. δH 1.12-1.2 (6H, m, 2 x CH_3), 1.36 (3H, d, J=6.4Hz, CH_3), 2.73 (1H, p, J=7.3Hz, **H-2**), 3.54 (3H, s, OMe), 3.99 (2H, m, OCH_2), 5.39, m, **H-3**), 7.41,7.51 (5H, 2 x br s, **phenyl**); m/z 317 (M^+-45, 2.1%), 216 (4.2), 189 (100), 115 (7.9), 73 (34.1).

Synthesis of the N-acetylcysteamine thioester of (5S, 4S, 2E)-5-hydroxy-4-methylhex-2-enoate (165).

a) To 4.72g (32.2mmol) of ethyl (2S, 3S)-3-hydroxy-2-methylbutanoate (**161**) in 10ml of ethoxyethene was added 1 drop of TFA. The mixture was left stirring overnight and the volatiles removed *in vacuo* to leave **ethyl (2S, 3S)-3-(1'-ethoxy)ethoxy-2-methyl butanoate** as a colourless oil (7.03g, 32.1mmol, 99%). υ_{max}(thin film) 1725 cm^{-1}; δ_H 1.08-1.3 (15H, m, 5 x CH_3), 2.44 (1H, m, $CHCH_3$), 3.38-3.67 (2H, m, OCH_2CH_3), 3.84 (1H, m, CHO), 4.15 (2H, m, OCH_2), 4.69 (1H, $OCH(CH_3)O$); m/z 203 (M^+-15, 1.1%), 173 (3.5), 129 (23.6), 73 (100), 45 (40.7).

b) To 0.5299g (13.94mmol) of lithium aluminium hydride suspended in 10ml of freshly distilled ether at 0°C under a nitrogen atmosphere was added ethyl (2S, 3S)-3-(1-ethoxy)ethoxy-2-methylbutanoate (3.039g, 13.94 mmol) dropwise as a solution in 5ml of anhydrous ether. The mixture was stirred at room temperature for one hour and then under reflux for two hours. After cooling to 0°C, the mixture was slowly decomposed by the dropwise addition of water. Upon completion ethyl acetate was added (20ml) and the mixture filtered with ethyl acetate washing. The organics were dried over anhydrous magnesium sulphate, filtered, concentrated and the residue purified by bulb to bulb distillation (77°C, 5mmHg) to yield **(2S, 3S)-3-(1'-ethoxy)ethoxy-2-methylbutanol (166)** (2.12g, 12.05mmol, 87%) as a colourless oil. υ_{max}(thin film) 3400, 1445, 1377cm^{-1}; δ_H 0.94, (3H, d, J=6.9Hz, CH_3CH), 1.2-1.35 (9H, m, 3 x CH_3), 3.4-3.8 (5H, m, 2 x CH_2O and H-3), 4.71 (1H, m, $OCH(CH_3)O$); m/z 116 (M^+-15, 0.6%), 131 (4.4%), 69 (26.1),73 (100), 45 (94.1).

c) To 25ml of dichloromethane at -78°C under a nitrogen atmosphere was added 1.20ml (13.75mmol) of oxalyl chloride. To this was added 1.95ml of dimethylsulphoxide (27.5mmol). Two minutes later (2S, 3S)-3-(1-ethoxy) ethoxy-2-methylbutanol **(166)** was added over two minutes as a solution in 3ml of dichloromethane. After a further 15 minutes triethylamine (17.49ml, 125mmol) was added, and after five minutes the mixture was allowed to warm to room temperature. Water (30ml) was added and the layers separated. The aqueous layer was was extracted with dichloromethane (2 x 30ml) and the combined organics dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The residue was taken up in 15ml of dichloromethane and washed sequentially with water, 5% sodium bicarbonate solution, water, 1% hydrochloric acid, and water. The solution was dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield **(2S, 3S)-3-(1'-ethoxy)ethoxybutanal (167)** (1.498g, 8.42mmol, 67%) as a pale straw oil υ_{max}(thin film) 1719 cm^{-1}; δ_H 1.0-1.4 (12H, m, 4 x CH_3).), 2.5 (1H, m, **H-2**), 3.4 (2H, m, CH_2CH_3), 4.0 (1H, m, CHOH), 4.75 (1H, m, $OCH(CH_3)O$), 9.77 (1H, s, CHO); m/z 175 (M^++1, 2.1%), 174 (M^+, 0.5%), 159 (1.1), 73 (97), 45 (100), 43 (31).

A) linear approach

a) A chloroform solution of (3S,2S)-3-(1'-ethoxy)ethoxy-2-methyl butanal (0.503g, 2.86mmol) (**167**) and the ethyl ester of triphenylphosphorane acetate was heated under reflux for 24 hours. After cooling the solvent was removed and the mixture purified by flash column chromatography (ethyl acetate/petrol 40-60, 4:1) to yield **ethyl (2E, 4S, 5S)-5-(1'-ethoxy)ethoxy-4-methylhex-2-enoate (168)** as a colourless oil (0.2719g, 1.11mmol, 39%). υ_{max}(thin film) 1719, 1654 cm^{-1}; δ_H 1.05-1.38 (15H, m, 3xCH_3), 2.47 (1H, m, **H-4**), 3.43-3.7 (3H, m, OCH_2 and **H-5**), 4.20 (2H, q, J=6.7Hz, OCH_2), 4.72 (1H, m, $OCH(CH_3O)$, 5.81 (1H, d, J=15.3Hz, **H-2**), 6.91 (1H, dd, J=15.3 Hz, J=3 Hz, **H-3**)

b) To ethyl (2E, 4S, 5S)-5-(1'-ethoxy)ethoxy-4-methylhex-2-enoate (**168**) (0.272g, 1.11mmol) was added 0.83ml of a 1.96M NaOH solution. This was stirred at room temperature overnight and then acidified to pH 1.0 and left stirring overnight. The mixture was extracted with ethyl acetate (5 x 10ml)

and the combined organics were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give **(2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoic acid** (0.070g, 0.44mmol, 45%) as a white solid. δ_H 1.1 (3H, d,J=6.9Hz, CH_3), 1.23 (3H, d, J=6.3Hz, CH_3), 2.41 (1H, m, **H-4**), 3.8 (1H, m ,**H-5**), 5.85 (1H, d, J=15.8 Hz, J=7.1 Hz, **H-2**), 7.13 (1H, dd, J=15.8 Hz, J=7.1Hz, **H-3**), 7.97 (2H, br s, 2 x OH).

c) To (2E, 4S, 5S)-5-hydroxy-2-methylhex-2-enoic acid (0.07g, 0.44mmol) in 3ml of dichloromethane under a nitrogen atmosphere was added 0.065g (0.54mmol) of freshly prepared N-acetylcysteamine (**98**) in 1ml of dichloro methane. To this was added DCC (0.117g, 0.45mmol) and two crystals of DMAP in 2ml of dichloromethane. The mixture was left overnight and then filtered through celite. After removal of the solvent *in vacuo* the residue was purified by preparative TLC (acetone/dichloromethane 2:3) to yield the **N-acetyl cysteamine thioester of (2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (165)** (0.072g, 0.29mmol, 59%) as a colourless syrup. υ_{max} ($CHCl_3$) 3299, 1659, 1625, 1559, 1551 cm^{-1}; δ_H 1.1 (3H, d, J=7.3 Hz, CH_3), 1.21 (3H, d, J=6.4 Hz, CH_3), 1.98 (3H, s, CH_3CON), 2.37 (1H, m, **H-4**), 3.09 (2H, t, J=6.5Hz, CH_2S), 3.46 (2H, m, CH_2N), 3.78 (1H, m, **H-5**), 6.07 (1H, br s, NH), 6.09 (1H, d, J=15.9 Hz, **H-2**), 1.92 (1H, dd, J=15.9 Hz, 6.9Hz, **H-3**); δ_C 14.16 (CH_3), 21.01 (CH_3), 23.29 (CH_2N), 28.43 (CH_2S), 39.77 ($COCH_3$), 40.04 (CHC=C), 70.76 (CHOH), 128.93 (C-2), 148.00 (C-3), 170.64 (CON), 190.46 (COS); m/z 201 (M^+-44, 7.7%), 119 (41.3), 83 (91.3), 82 (63.5), 55 (100), 45 (65.2)

B) convergent approach

To (2S,3S)-3-(1'-ethoxy)ethoxy-2-methylbutanal (**167**) (0.2417g, 1.37 mmol) in 10ml of chloroform was added the N-acetylcysteamine thioester of triphenyl phosphorane acetate (**107**) (0.578g, 1.37mmol). The mixture was heated under reflux for 18 hours. The solvent was removed and the residue taken up in 10ml of methanol. To this was added 1 drop of trifluoroacetic acid and the mixture left stirring overnight. The volatiles were removed and the residue purified by flash column chromatography (acetone/dichloromethane, 2:3) to yield the **N-acetylcysteamine thioester of (2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (165)** (0.197g, 0.814 mmol, 59%) as a colourless syrup. Spectroscopic data as above

Synthesis of the N-acetylcysteamine thioester of [7-$^2H_3$]-(5S, 4S, 2E) -5-hydroxy-4-methylhex-2-enoate (165).

a) To 0.931g (6.21mmol) of ethyl [5-$^2H_3$]-(2S, 3S)-3-hydroxy-2-methyl butanoate (**161**) in 5ml of ethoxyethene was added 1 drop of TFA. The mixture was left stirring overnight and the volatiles removed *in vacuo* to leave **ethyl [5-$^2H_3$]-(2S, 3S)-3-(1'-ethoxy)ethoxy-2-methyl butanoate** as a colourless oil (1.263g, 6.21mmol, 100%). υ_{max}(thin film) 1725 cm^{-1}; δ_H 1.08-1.3 (12H, m, 4 x CH_3), 2.44 (1H,br m, CHC^2H_3), 3.38-3.67 (2H, m, OCH_2CH_3), 3.84 (1H, m, CHO), 4.15 (2H, m, OCH_2), 4.69 (1H, $OCH(CH_3)O$); m/z 203 (M^+-18, 0.6%), 202 (2.3), 149 (5.3), 84 (42.8), 73 (77.1), 45 (100).

b) To 0.196g (5.1mmol) of lithium aluminium hydride suspended in 10ml of freshly distilled ether at 0°C under a nitrogen atmosphere was added ethyl [5-$^2H_3$]-(2S, 3S)-3-(1-ethoxy)ethoxy-2-methylbutanoate (1.045g, 5.10mmol) as a solution in 5ml of anhydrous ether. The mixture was stirred at room temperature for one hour and then under reflux for two hours. After cooling to 0°C, the mixture was slowly decomposed by the dropwise addition of water. Upon completion ethyl acetate was added (20ml) and the mixture filtered with ethyl acetate washing. The organics were dried over anhydrous magnesium sulphate, filtered, concentrated and the residue purified by bulb to bulb distillation (77°C, 5mmHg) to yield **[5-$^2H_3$]-(2S, 3S)-3-(1'-ethoxy)ethoxy-2-methylbutanol (166)** (0.685g, 4.23mmol, 83%) as a colourless oil. υ_{max}(thinfilm) 3394, 1445, 1375cm^{-1}, δ_H 1.2-1.35 (9H, m, 3 x CH_3), 1.64 (1H, br s, OH) 3.4-3.8 (5H, m, 2 x CH_2O and H-3), 4.71 (1H, m, $OCH(CH_3)O$); m/z 161 (M^+-18, 0.9%), 73 (100), 45 (89.4).

c) To 15ml of dichloromethane at -78°C under a nitrogen atmosphere was added 0.59ml (4.6mmol) of oxalyl chloride. To this was added 0.72g of dimethylsulphoxide (9.19mmol). Two minutes later [5-$^2H_3$]-(2S, 3S)-3-(1'-ethoxy) ethoxy-2-methylbutanol (**166**) (0.678g, 4.18mmol) was added over two minutes as a solution in 3ml of dichloromethane. After a further 15 minutes triethylamine (4.22g, 41.8mmol) was added, and after five minutes the mixture was allowed to warm to room temperature. Water (20ml) was added and the layers separated. The aqueous layer was extracted with dichloromethane (2 x 20ml) and the combined organics dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. The

residue was taken up in 15ml of dichloromethane and washed sequentially with water, 5% sodium bicarbonate solution, water, 1% hydrochloric acid, and water. The solution was dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to yield **[5-$^2H_3$]-(2S, 3S)-3-(1'-ethoxy)ethoxybutanal (167)** (0.439g, 2.67mmol, 64%) as a pale straw oil. υ_{max}(thin film) 1721 cm^{-1}; δ_H 1.0-1.4 (9H, m, 3 x CH_3).), 2.5 (1H, m, **H-2**), 3.4 (2H, m, CH_2CH_3), 4.0 (1H, m, CHOH), 4.75 (1H, m, $OCH(CH_3)O$), 9.77 (1H, s, CHO); m/z 159 (M^+-15, 1.1%), 73 (97), 45 (100), 43 (31).

d) To [5-$^2H_3$]-(2S, 3S)-3-(1'-ethoxy)ethoxy-2-methylbutanal **(167)** (0.2417g, 1.37mmol) in 10ml of chloroform was added the N-acetylcysteamine thioester of triphenyl phosphorane acetate **(107)** (0.578g, 1.37mmol). The mixture was heated under reflux for 18 hours. The solvent was removed and the residue taken up in 10ml of methanol. To this was added 1 drop of trifluoroacetic acid and the mixture left stirring overnight. The volatiles were removed and the residue purified by flash column chromatography (acetone/dichloromethane, 2:3) to yield the **N-acetylcysteamine thioester of [7-$^2H_3$]-(2E, 4S, 5S)-5-hydroxy-4-methylhex-2-enoate (165)** (0.197g, 0.814 mmol, 59%) as a colourless syrup. υ_{max}($CHCl_3$) 3301, 1659, 1624, 1560, 1459 cm^{-1}; δ_H 1.21 (3H, d, J=6.4 Hz, CH_3), 1.98 (3H, s, CH_3CON), 2.37 (1H, m, **H-4**), 3.09 (2H, t, J=6.5Hz, CH_2S), 3.46 (2H, m, CH_2N), 3.78 (1H, m, **H-5**), 6.07 (1H, br s, NH), 6.09 (1H, d, J=15.9 Hz, **H-2**), 1.92 (1H, dd, J=15.9 Hz, 6.9Hz, **H-3**); δ_C 21.05 (CH_3), 23.27 (CH_2N), 28.44 (CH_2S), 39.73 (CH_3CON), 40.04 (CHC=C), 70.76 (CHOH), 128.89 (C-2), 148.04 (C-3), 170.66 (CON), 190.48 (COS); δ_D 1.05 (br s); m/z 204 (M^+-44, 12.2%), 119 (47.9), 58 (100), 43 (77).

Synthesis of disodium 2-methylmalonate (176)

a) To 0.2571g of sodium under a nitrogen atmosphere at 0°C was added 10ml of absolute ethanol. Once dissolution was complete diethyl malonate **(73)** (1.5g, 9.32mmol) was added and the mixture left 30 minutes. Iodomethane (1.998g, 13.98mmol) was added and the mixture left 30 more minutes. Water (5ml) was added and the solvents removed *in vacuo*. The residue was taken up in 10ml of ether and washed for 1 minute with 30% (w/v) sodium hydroxide solution. The ethereal solution was dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. Bulb to bulb distillation (72°C, 6mmHg) gave **diethyl 2-methylmalonate** (1.371g,

7.57mmol,81%) as a colourless oil. υ_{max}(thin film) 2991, 1728 cm^{-1}; δ_H 1.15 (3H, t, J=6.7 Hz, CH_2CH_3), 1.42 (3H, d, J≈7Hz, CH_3CH), 3.42 (1H, q, J=7Hz, $CHCH_3$), 4.2 (2H, q, J=6.7Hz, OCH_2CH_3).

b) To 0.873g (5.02mmol) of diethyl 2-methylmalonate was added 5.25ml of 1.96M NaOH solution. This was stirred overnight. The water was removed *in vacuo* and the residue freeze dried to leave 0.811g (5.01mmol,100%) of **disodium 2-methylmalonate (176)** as a white solid. υ_{max}(nujol) 1551, 1449 cm^{-1}; $\delta_H(^2H_2O)$ 1.39 (3H, dq, J=7.1Hz, CH_3CH), 3.17 (1H, q, J=7.1Hz, $CHCH_3$).

Synthesis of disodium [2,4-$^{13}C_2$]-methylmalonate (176)

a) To 0.2571g of sodium under a nitrogen atmosphere at 0°C was added 10ml of absolute ethanol. Once dissolution was complete diethyl [2-^{13}C]-malonate (1.5g, 9.32mmol) was added and the mixture left 30 minutes. [^{13}C]-Iodomethane (1.998g, 13.98mmol) was added and the mixture left 30 more minutes. Water (5ml) was added and the solvents removed *in vacuo*. The residue was taken up in 10ml of ether and washed for 1 minute with 30% (w/v) sodium hydroxide solution. The ethereal solution was dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo*. Bulb to bulb distillation (72°C, 6mmHg) gave **diethyl [2,4-$^{13}C_2$]-methylmalonate** (1.371g, 7.57mmol,81%) as a colourless oil. υ_{max}(thin film) 2987, 1725 cm^{-1};δ_H 1.15 (6H, t, J=6.6Hz, CH_3CH_2), 1.42 (3H, dm, $^1J_{CH}$=132Hz, CH_3CH), 3.2 (1H, dm, $^1J_{CH}$=132Hz, $CHCH_3$), 4.2 (4H, q, J=6.6Hz, OCH_2)

b) To 0.338g (1.925mmol) of diethyl [2,4-$^{13}C_2$]-methylmalonate was added 2.01ml of 1.96M NaOH solution. This was stirred overnight. The water was removed *in vacuo* and the residue freeze dried to leave 0.315g (1.921, 99%) of **disodium [2,4-$^{13}C_2$]-methylmalonate (176)** as a white solid. υ_{max}(nujol) 1550, 1450 cm^{-1}; $\delta_H(^2H_2O)$ 1.39 (3H, dm, $^1J_{CH}$=134Hz, CH_3CH), 3.17 (1H, dm, $^1J_{CH}$=134Hz, $CHCH_3$).

Synthesis of serine (169)

To an aqueous suspension (1ml) of diethyl acetamidomalonate (**170**) (1.154g, 5.32mmol) was added 0.46ml of 40% formalin (5.85 mmol) and 1 drop of 2M NaOH. The mixture was stirred for two hours by which time a colourless solution had formed. To this was added 5.6ml of 2M NaOH (11.5mmol) and the reaction left stirring overnight. Acetic acid (3.3ml) was added and the mixture concentrated over a steam cone. To the resultant syrup was added 3ml of concentrated HCl and the mixture heated under reflux for 1 hour. The water was removed *in vacuo* and the resultant solid residue leached with boiling absolute ethanol. The ethanolic solution was concentrated and the residual solid taken up im 3.5 ml of 2M NaOH. This was left stirring overnight. Water was added and the pH adjusted to 7.0. The mixture was treated with activated charcoal and filtered. The water was removed *in vacuo* to leave a cream solid. This re-dissolved in 3ml of water and an excess of hot absolute ethanol was added. After cooling overnight the white solid was freeze dried to leave 0.395g (3.79mml, 61%) of **serine (169)**. υ_{max}(nujol) 1655, 1635, 1577 cm^{-1}; $\delta_H(^2H_2O)$ 3.83 (1H, ,m, $CHCH_2$), 3.94 (2H, m, CH_2CH); δ_C 56.29 (CH), 60.07 (CH_2), 172.29 (CO_2H).

Synthesis of [3-^{13}C]-serine (169)

To an aqueous suspension (1ml) of diethyl acetamidomalonate (**170**) (1.154g, 5.32mmol) was added 1ml of 20% [^{13}C]-formalin (5.85 mmol) and 1 drop of 2M NaOH. The mixture was stirred for two hours by which time a colourless solution had formed. To this was added 5.6ml of 2M NaOH (11.5mmol) and the reaction left stirring overnight. Acetic acid (3.3ml) was added and the mixture concentrated over a steam cone. To the resultant syrup was added 3ml of conc. HCl and the mixture heated under reflux for 1 hour. The water was removed *in vacuo* and the resultant solid residue leached with boiling absolute ethanol. The ethanolic solution was concentrated and the residual solid taken up in 3.5 ml of 2M NaOH. This was left stirring overnight. Water was added and the pH adjusted to 7.0. The mixture was treated with activated charcoal and filtered. The water was removed *in vacuo* to leave a cream solid. This re-dissolved in 3ml of water and an excess of hot absolute ethanol was added. After cooling overnight the white solid was freeze dried to leave 0.395g (3.79mml, 61%) of **[3-^{13}C]-serine (169)**. υ_{max}(nujol) 1654, 1635, 1576 cm^{-1}; $\delta_H(^2H_2O)$ 3.83 (1H, m , $CHCH_2$), 3.94 (2H, dm, $^1J_{CH}$=141Hz, CH_2CH).

Incorporation studies

a) The N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-acetoacetate (**144**) plus tetradecylthiopropionic acid (**89**)

Primary and secondary media were inoculated as previously described. A 10ml solution (DMSO) containing the N-acetylcysteamine thioester of [2,3-$^{13}C_2$]-acetoacetate (**144**) (207mg) plus tetradecylthiopropionic acid (**87**) (30mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 14mg of methyl pseudomonate (**58**) were isolated. The ^1H nmr spectrum was identical to that of an authentic sample.

b) The N-acetylcysteamine thioester of [5-$^2H_3$]-(2S,3S)-3-hydroxy-2-methyl buatanoate (**159**) plus tetradecylthiopropionic acid (**89**)

Primary and secondary media were inoculated as previously described. A 10ml solution (DMSO) containing The N-acetylcysteamine thioester of [5-$^2H_3$]-(2S,3S)-3-hydroxy-2-methylbuatanoate (**159**) (243mg) plus tetradecyl thiopropionic acid (**89**) (30mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 12mg of methyl pseudomonate (**58**) were isolated. Also isolated was 11mg of the N-acetylcysteamine thioester of [5-$^2H_3$]-(2S,3S)-3-hydroxy-2-methylbutanoate (**159**). The ^1H nmr spectrum was identical to that of an authentic sample. δ_D 1.21, 2.57, 3.73.

c) The N-acetylcysteamine thioester of [7-$^2H_2$]-(2E,4S,5S)-5-hydroxy-4-methylhex-2-enoate (**165**) plus tetradecylthiopropionic acid (**89**)

Primary and secondary media were inoculated as previously described. A 10ml solution (DMSO) containing The N-acetylcysteamine thioester of [7-$^2H_2$]-(2E,4S,5S)-5-hydroxy-4-methylhex-2-enoate (**165**) (197mg) plus tetradecylthiopropionic acid (**89**) (30mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 11mg of methyl pseudomonate (**58**) were isolated.The ^1H nmr spectrum was identical to that of an authentic sample. δ_D 1.22, 3.76.

d) [3-^{13}C]-Serine (**169**)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing [3-^{13}C]-serine (**169**) (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 3mg of methyl pseudomonate (**58**) were isolated.The ^{1}H nmr spectrum was identical to that of an authentic sample.

e) Sodium [2-^{13}C]-acetate

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing Sodium [2-^{13}C]-acetate (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. At the end of the growth period, the media pH was 4.75, indicating that the growth cycle had not been completed. No methyl pseudomonate was isolated.

f) Disodium [4-^{13}C]-2-methylmalonate (**176**)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing disodium [4-^{13}C]-2-methylmalonate (**176**) (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and 22mg of methyl pseudomonate (**58**) were isolated.The ^{1}H and ^{13}C nmr spectra were identical to that of an authentic sample.

g) Disodium [2,4-$^{13}C_2$]-2-methylmalonate (**176**)

Primary and secondary media were inoculated as previously described. A 10ml solution (H_2O) containing disodium [2,4-$^{13}C_2$]-2-methylmalonate (**176**) (250mg) was prepared. 5ml of this was added to the secondary medium (0.5ml per flask) seventeen hours after inoculation. This was repeated after a further three hours. The cells were harvested as usual, and

25mg of methyl pseudomonate (58) were isolated.The ^{1}H and ^{13}C nmr spectra were identical to that of an authentic sample.

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